Universidade de Aveiro Departamento de Biologia
Ano 2015

PAULA DA SILVA TOURINHO

EFEITOS DE NANOPARTICULAS METÁLICAS NO ISÓPODE TERRESTRE *Porcellionides pruinosus*

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora auxiliar do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar e co-orientação do Doutor Cornelis Adrianus Maria van Gestel, Professor Associado do Departamento de Ecologia Animal da Faculdade de Ciências da Terra e da Vida da Universidade Livre de Amesterdão, Holanda.

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Aos meus pais

“The most alarming of all man’s assaults upon the environment is the contamination of air, earth, rivers, and sea with dangerous and even lethal materials. This pollution is for the most part irrecoverable; the chain of evil it initiates not only in the world that must support life but in living tissues is for the most part irreversible.”

Rachel Carson

o júri
presidente

Prof. Doutor Jorge Ribeiro Frade
Professor Catedrático da Universidade de Aveiro

Doutor Nicolaas Wilhelmus van den Brink
Professor Associado da Universidade de Wageningen, Holanda

Doutor Claus Svendsen
Investigador Principal do Centro de Ecologia e Hidrologia, Reino Unido

Doutor Tiago Manuel Ferreira Natal da Luz
Investigador em Pós-Doutoramento da Universidade de Coimbra

Doutora Isabel Maria Cunha Antunes Lopes
Investigadora Principal da Universidade de Aveiro

Prof. Doutor Amadeu Mortágua Velho da Maia Soares
Professor Catedrático da Universidade de Aveiro

Doutor Cornelis Adrianus Maria van Gestel (co-orientador)
Professor Associado da Universidade Livre de Amsterdão, Holanda

Prof. Doutora Susana Patrícia Loureiro (orientadora)
Professora Auxiliar da Universidade de Aveiro

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palavras-chave Nanopartículas, *Porcellionides pruinosus*, Bioacumulação, Ecotoxicologia.

resumo

Nanopartículas (NPs) apresentam propriedades intrínsecas que são distintas de outros materiais. Como consequência, a produção de nanopartículas tem aumentado nos últimos anos, principalmente utilizadas em produtos de consumo. Devido a sua libertação de produtos, as NPs podem estar presentes no ambiente, incluindo os compartimentos terrestres. No entanto, a toxicidade das NPs não está completamente compreendida, e há poucos estudos realizados em exposições terrestres. Portanto, o objetivo desta tese é aumentar o conhecimento sobre os efeitos tóxicos das NPs no solo. Para isso, uma revisão da literatura sobre o destino e a toxicidade de nanopartículas metálicas para invertebrados terrestres foi incluída. A seguir, a toxicidade de três nanopartículas metálicas, nomeadamente óxido de zinco (ZnO), prata (Ag), e dióxido de cério (CeO_2), foi avaliada no isópode terrestre *Porcellionides pruinosus*.

O pH do solo afetou a toxicidade do ZnO e cloreto de zinco ($ZnCl_2$), que foi geralmente menor no pH intermediário (pH ~5-6). As fracções dissolvidas de Zn (Zn na água intersticial e iões livre) não pôde explicar a toxicidade, o que sugere uma contribuição das formas não dissolvidas de Zn. Além disso, a acumulação de Zn através do ZnO e $ZnCl_2$ não foi afetada pelo pH do solo. Em relação as nanopartículas de Ag (Ag NPs), o comportamento de evitamento mostrou ser teste mais sensível e não houve diferença entre Ag NPs e nitrato de prata ($AgNO_3$). No entanto, no teste de inibição da alimentação, a forma iónica causou maior perda de biomassa nos isópodes quando exposto através do solo e alimento. Comparando ambas vias de exposição, o solo resultou em maior toxicidade e maior concentração de Ag nos isópodes. A exposição pelo solo também resultou em taxas de acumulação e eliminação semelhantes entre Ag NPs e $AgNO_3$, mostrado pelo estudo de bioaccumulação. A exposição ao alimento contaminado resultou em menores taxas de acumulação, quando comparado ao solo, mas as constantes de velocidade de eliminação foram similares. Os resultados da toxicocinética e imagens de raios-X por radiação de sincrotrão sugeriram que a Ag proveniente de Ag NPs é armazenada nas células-S no hepatopâncreas e praticamente não são eliminadas. Por último, as nanopartículas de CeO_2 não mostraram efeitos na sobrevivência e na biomassa. Além disso, a toxicidade combinada de CeO_2 e fenantreno mostrou que o CeO_2 não altera a toxicidade do fenantreno nos isópodes.

Os resultados desta tese demonstram a relevância em se estudar a toxicidade das NPs, como partículas nas dimensões nano também foram biodisponível para os isópodes, e não apenas as frações dissolvidas. Além disso, o isópode *Porcellionides pruinosus* se mostrou um organismo adequado para avaliar a toxicidade NP no solo.

keywords Nanoparticles, *Porcellionides pruinosus*, Bioaccumulation, Ecotoxicology

abstract

Nanoparticles (NPs) present intrinsic properties that distinguish them among other materials. As a consequence, the production of nanoparticles has increased over the last years, being especially used in consumer products. Due to their release from NP-containing products, NPs will enter the environments, including the terrestrial compartments. Nevertheless, the toxicity of NPs is not completely understood, and up-to-date, there is a lack of studies undertaken in soil exposures. Therefore, the aim of this thesis is to increase the knowledge on the toxic effects of NPs in the soil compartment. For that, a literature review of fate and toxicity of metal-based NPs to soil invertebrates was included. Then, the toxicity of three metal-based nanoparticles, namely zinc oxide (ZnO), silver (Ag) and cerium oxide (CeO_2), was assessed on the terrestrial isopod *Porcellionides pruinosus*.

Soil pH affected the toxicity of ZnO and zinc chloride ($ZnCl_2$), which was generally lower at intermediate pH (pH ~5 to 6). The dissolved fractions of Zn (porewater Zn and free Zn ion) could not explain the toxicity, suggesting a contribution of non-dissolved Zn forms. Regarding Ag NPs, avoidance behavior showed to be most sensitive endpoint and no difference was found between Ag NPs and silver nitrate ($AgNO_3$). However, in the feeding inhibition test, ionic Ag caused greater biomass loss in isopods when exposed via soil and food. Comparing both routes, soil exposure resulted in greater toxicity and higher Ag body concentration in isopods. Soil exposure also resulted in similar uptake and elimination rate constants between Ag NPs and $AgNO_3$, shown by the kinetics study. Dietary exposure resulted in lower uptake rate constants when compared to soil, but elimination rate constants did not differ. Toxicokinetic results and synchrotron X-ray images suggested that Ag from Ag NPs is stored in the S-cells in the hepatopancreas and are hardly eliminated. Lastly, CeO_2 NPs showed no effects on survival and biomass change. Moreover, a combined toxicity of CeO_2 NPs and phenanthrene showed that CeO_2 NPs did not shift phenanthrene toxicity to isopods.

The outcomes of this thesis demonstrate the relevance of studying NP toxicity, as nanosized particles were also bioavailable to isopods, and not only the dissolved fractions. Furthermore, the isopod *Porcellionides pruinosus* was found to be a suitable model organism to assess NP toxicity in soil.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Scope

For many years, nanomaterial or nanoparticles (NPs) were defined as any natural or manufactured material presenting 1 to 100 nm at least in one of their dimensions (Lead and Wilkinson, 2006; Oberdörster, 2004). As the scientific knowledge and regulatory concern increased, a more specific definition was found to be necessary. In 2011, the European Commission introduced a new definition for a nanoparticle, as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm" (European Union, 2011).

Nanoparticles have drawn attention due to their intrinsic physico-chemical properties, especially the large surface area to volume ratio and high surface reactivity (Handy et al., 2008). In accordance to the new definition, the ratio surface area: volume of NPs must be ≥ 60 m^2/cm^3 (European Union, 2011). Due to these unique properties, a great variability of applications for NPs has been developed, resulting in a massive increase of the 'nanotechnology' industry over the years. Among the most used NPs nowadays, metal-based (i.e., metal and metal oxide) nanoparticles stand out due to the high production worldwide, especially TiO_2, ZnO, FeOx, AlOx, CeO_2, and Ag NPs (Piccinno et al., 2012). Recent studies have highlighted the environmental exposure to metal-based NPs, as their production continuously increases (Gottschalk et al., 2013; Sun et al., 2014).

This thesis focuses on the toxicity of three metal-based nanoparticles, namely zinc oxide (ZnO), silver (Ag) and cerium oxide (CeO_2) nanoparticles. To assess their toxicity, the terrestrial isopod *Porcellionides pruinosus* was chosen as the model species.

In this chapter, the latest findings on the fate and toxicity of metal-based nanoparticles in soil are described. Then, the importance of isopods as model species is presented. And finally, the aim and description of the following chapters is given.

1.2 Metal-based nanoparticles: fate and toxicity in soils

Nowadays, NPs are used in diverse consumer products, including cosmetics and personal care products, textiles, coatings, paints, plastics, and others (Gottschalk et al., 2013; Piccinno et al., 2012). The use of NP-containing products results in their release to the environment, primarily reaching the wastewater treatment plants (WWTPs) (Benn et al., 2010; Kaegi et al., 2011). In the WWTPs, NPs may be distributed over effluents and sewage sludge (Kaegi et al., 2011). Through the land application of biosolids as fertilizers, NPs may end up in the soil compartment, which is considered their main route into soils (Gottschalk et al., 2009). Thus, understanding NP fate in soil and their toxicity to soil organisms is essential.

1.2.1 Fate of metal-based NPs in soil

The characteristics of NPs (e.g., size, coating, surface charge) and soil properties (e.g., pH, ionic strength, organic matter and clay content) are well known to affect NP behavior in soils (Cornelis et al., 2012; Darlington et al., 2009; Fang et al., 2011; Sagee et al., 2012). These properties will affect the processes of NP dissolution and aggregation/agglomeration. For instance, the dissolution of ions from NPs is dependent on the particle size and on the surface area, and therefore the larger the particles are, the lower their dissolution rate (Coutris et al., 2012). At the same time, higher organic matter content in soil or soil solution leads to reduced release of metal ions due to sorption of organic matter components on the NP surface (Cornelis et al., 2012; Klitzke et al., 2014; Kühn et al., 2014).

Many studies have been focused on the behavior of NPs not only in soil, but also in the sludge in WWTPs and in biosolids. By modeling their fate in WWTPs, Barton et al. (2015) found that CeO_2 NPs were mainly found in the biosolids rather than in the effluents. It suggests that CeO_2 NPs are very likely to enter the soil compartment through the land application of biosolids. For Ag NPs, low dissolution and transformation into Ag_2S was observed in biosolids (Lombi et al., 2013; Ma et al., 2013; Navarro et al., 2014) and sewage sludge (Lombi et al., 2013; Ma et al., 2013; Whitley et al., 2013). Similarly, low oxidation/dissolution of Ag NPs was observed in artificial (Gomes et al., 2013; Shoults-Wilson et al., 2011) and natural

soils (Cornelis et al., 2012; Waalewijn-Kool et al., 2014), as well as in sludge amended soils (Whitley et al., 2013).

More recent studies have been also focused on the effects of aging on the fate of NPs in soil. Different behavior of uncoated and coated NPs was observed in soil for Ag (Coutris et al., 2012; Whitley et al., 2013) and ZnO NPs (Waalewijn-Kool et al., 2013). However, NP fate in soil is quite complex being influenced by other soil properties, which may also change with time. Citrate coated Ag NPs showed similar dissolution rate as $AgNO_3$ in organic soil, while in mineral soil $AgNO_3$ showed higher dissolution after 70 days (Coutris et al., 2012). Uncoated Ag NPs, however, were less affected by soil type (Coutris et al., 2012). Still, considering a longer time period, different conclusions may be found. In the study of Whitley et al. (2013), after a 6-month period, dissolved Ag in pore water was < 2% of total Ag in soil for both citrate- and PVP-coated Ag NPs and ionic Ag as $AgNO_3$. Similar levels of Ag dissolution were also observed between Ag NPs (sodium polyacrylate coated) and $AgNO_3$ after 6 months (Benoit et al., 2013). For uncoated and triethoxyoctylsilane-coated ZnO NPs and non-nano ZnO, Zn porewater concentrations increased over 12 months (Waalewijn-Kool et al., 2013). Zn dissolution was not dose-related with total Zn concentration in soil, which could be explained by higher soil pH at higher ZnO NP concentrations (Waalewijn-Kool et al., 2013). When comparing ZnO NPs and ionic Zn as $ZnCl_2$, much higher Zn levels in porewater were observed for $ZnCl_2$.

1.2.2 Toxicity of metal-based NPs to soil organisms

Assessing the toxicity of NPs to soil organisms is a challenging subject. As described above, a number of processes may occur with NPs once they enter terrestrial environments and these processes may have influence on their toxicity. Moreover, adjustments to the current practices of the toxicity tests may be necessary in the case of nanoparticles (van Gestel, 2012). For instance, different spiking procedures (e.g., addition as dry-powder or as solution) could affect toxicity outcomes and should be investigated (Waalewijn-Kool et al., 2012).

In the OECD Expert Meeting Report on “Ecotoxicology and Environmental fate of Manufactured Nanomaterials: Test Guidelines”, several guidelines are described

as being applicable to ENMs (engineered nanomaterials), considering the current practices available. Among them, the OECD test guidelines (TG) 222 (earthworm reproduction) and 317 (Bioaccumulation with terrestrial Oligochaetes) are considered suitable to be used for NP assessment. On the other hand, there are other guidelines advised to be adapted or even denominated as non-appropriate for ENMs (TG 105 and 106). A specific TG for addressing the solubility of NPs in soils is necessary, since TG 105 is not appropriate; while the current TG 106 for adsorption/desorption behavior cannot be applied to NPs, as no valid methodology is available. Moreover, no final recommendation on the procedure for spiking test substrates with NPs was provided, since both procedures (i.e., dry and wet spiking) have pros and cons, and not enough data have been generated (OECD, 2014).

In soils, the dissolved forms of compounds are normally considered the bioavailable and consequently the toxic fraction. Thus, NP toxicity is usually compared with that of its ionic counterparts to better understand the role of the nanosized particles. Many studies have found that toxicity is caused by the release of metal ions from NPs (Gomes et al., 2013; Tsyusko et al., 2012). Nevertheless, a particle effect cannot always can be excluded, even though most of the toxicity may be driven by the dissolved forms (Heggelund et al., 2014). More research on the bioavailability and toxicity of NPs seems to be necessary to define to what extent toxicity is caused by the ions released from NPs.

No relation between the toxicity and body concentrations in test animals was observed for Ag (Diez-Ortiz et al., 2015; Schlich et al., 2013) and ZnO NPs (García-Gómez et al., 2014; Heggelund et al., 2014). It suggests that the relationship between NP toxicity and uptake is not completely straightforward, and complex processes and mechanisms of intoxication are probably involved.

1.3 Isopods as test organism

Terrestrial isopods are generalist macro-decomposers living in the top layer of terrestrial systems. The ecological relevance of this group is well described in the literature. They play an important role in soil ecosystem function such as organic matter decomposition, nutrient recycling (Kautz and Topp, 2000), the

fragmentation of litter (Hassall et al., 1987), distribution of nutrients and minerals (Hassall et al., 1987), and stimulate microbial activity (Hättenschwiler and Bretscher, 2001).

Another reason that makes isopods quite interesting test organisms is their ability of accumulating high levels of metals (Donker, 1992; Hopkin, 1990; Hopkin and Martin, 1982; Odendaal and Reinecke, 1999). This is only possible due to their hepatopancreas, which can accumulate up to 90% of total body metal concentration (Hopkin and Martin, 1982). The hepatopancreas is composed by two cell types, namely B (big) and S (small) cells. The B cells are responsible for absorption and secretion of metals, while the only function of S cells is absorption (Clifford and Witkus, 1971; Hames and Hopkin, 1989). While the S cells have a long residence time, the B cells undergo a diurnal cycle, voiding their content every 11 hours. Metal sequestration is also different in both cells, with S cells containing Cu and S-rich granules and B cells Fe and PO43- rich inclusions. At the same time isopods accumulate high levels of metal in their body, sublethal responses can be observed relatively fast (Drobne, 1997; Drobne and Hopkin, 1994; Drobne and Hopkin, 1995). Moreover, they can also be used as bioindicators of metal pollution, being suitable for assessing metal uptake from soil (Hopkin et al., 1986; Paoletti and Hassall, 1999).

The isopod *Porcellionides pruinosus* (Brandt 1833) stands out as one of the most cosmopolitan isopod species (Schultz, 1972), and inhabit near and benefit from anthropogenic activities. They have been used as model organism in many toxicity studies (Jänsch et al., 2005; Loureiro et al., 2006; Loureiro et al., 2005; Santos et al., 2010; Silva et al., 2014). Moreover, this species can be used in toxicity tests using both soil and dietary exposure routes, as shown by Sousa et al. (2000) and Vink et al. (1995).

Figure 1.1: Adult specimen of the isopod *Porcellionides pruinosus*.

1.4 Conceptual framework

Due to increasing production and use of NP-containing products, it is very likely that NPs will enter the soil environment, therefore raising concern about their hazards and safe levels to organisms. The main aim of this thesis is to evaluate the toxicity of ZnO, Ag and CeO_2 NPs to the isopod *Porcellionides pruinosus*. Data on the effects of NPs to isopods are lacking, especially considering soil as an exposure route.

The toxicity of ionic Zn to terrestrial isopods is very well known (Donker et al., 1998; Donker et al., 1996; Hopkin and Hames, 1994; Hopkin et al., 1986; Hopkin and Martin, 1982; Van Straalen et al., 2005; Vijver et al., 2006). Thus the toxicity of ZnO NPs was evaluated in different soils in order to investigate the influence of soil properties on ZnO NP toxicity. For Ag and Ce, no data on the toxicity to isopods was available, neither for nanosized particles nor for their ionic form. For Ag NPs, a deeper investigation on their toxic effects was chosen, since Ag was found to be one of the most toxic NPs (see review in Chapter 2). Therefore not only toxicity tests using soil and food exposures, but also toxicokinetic tests were conducted. CeO_2 NPs showed very low toxicity in a previous study (Lahive et al., 2014), it therefore was focused on its combined toxicity with phenanthrene, a compound that may be emitted together with CeO_2 NPs from diesel exhaust.

The specific aims of the thesis include: (i) assessing the effects of soil pH on the toxicity and uptake of ZnO NPs in isopods (Chapter 3); (ii) determining the bioavailability of ZnO and Ag NPs by assessing body concentrations or bioaccumulation kinetics (Chapter 3 to 5); (iii) comparing the toxicity and uptake of ZnO and Ag NPs to that of the ionic (Chapter 3 to 5) and non-nano forms (Chapter

3); assessing soil and dietary exposures to Ag NPs, evaluating different route of exposure (Chapters 4 and 5); and (v) determining whether CeO_2 NPs can shift the toxicity of phenanthrene (Chapter 6).

The thesis is structured in seven chapters, described in detail below.

In this first chapter (**Chapter 1**), a general introduction to the topic is provided, followed by **Chapter 2**, where a review of the literature on the fate and behavior of metal-based NPs and their toxicity to soil invertebrates is presented. The available knowledge and also the gaps and limitations of the field were discussed and used as baselines to explore the work carried out is this thesis.

Chapters 3 to 6 investigate the toxicity and toxicokinetic of the 3 metal-based NPs. In Figure 1.2, the approaches used in each chapter can be found.

In **Chapter 3**, the influence of soil pH on the toxicity of ZnO NPs, non-nano ZnO and $ZnCl_2$ to isopods was evaluated. A natural soil amended with different levels of $CaCO_3$, resulting in three different soil pHs, and the natural Lufa 2.2 soil were used in feeding inhibition tests. Survival, food consumption and biomass change were the endpoints assessed, and total Zn body concentration was determined.

In **Chapter 4**, the toxicity of Ag NPs and $AgNO_3$ was observed in isopods exposed to Lufa 2.2 soil and alder leaves as food. Avoidance behavior and feeding inhibition tests were performed and total Ag body concentration was measured.

In **Chapter 5**, the toxicokinetics of Ag NPs and $AgNO_3$ was investigated upon soil and dietary exposures (Lufa 2.2 soil and alder leaves, respectively). Uptake and elimination rate constants were determined using a one-compartment model and compared for both exposure routes.

In **Chapter 6**, the effect of CeO_2 (used as diesel catalyst) on the toxicity of phenanthrene (one of the major components in diesel exhaust emissions) was studied in a full-factorial design. Survival, food consumption and biomass change were observed and compared between treatments.

In **Chapter 7**, a general discussion of the results obtained in the previous chapters and a final conclusion are provided.

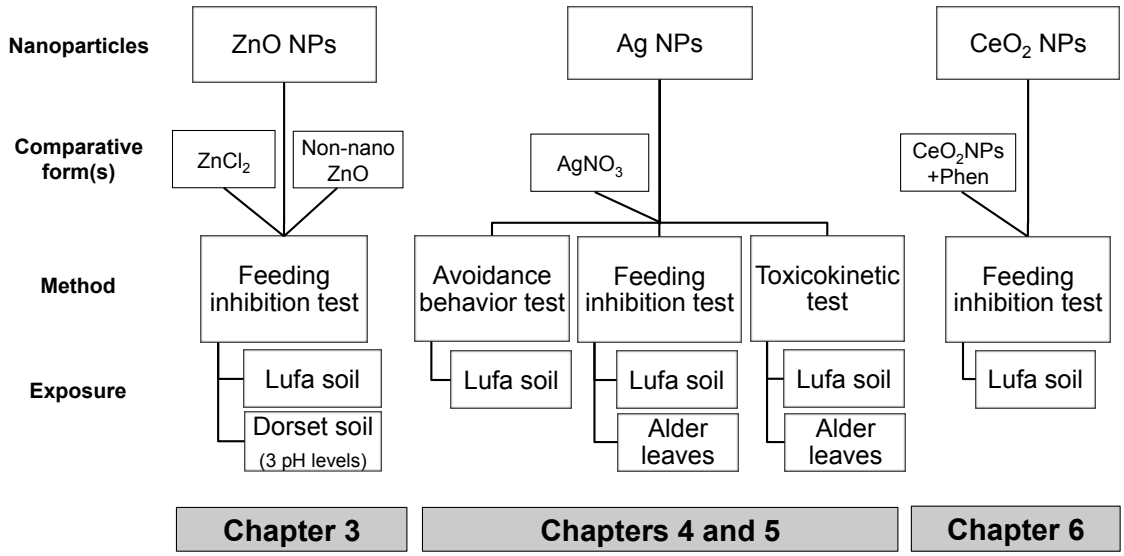

Figure 1.2: Scheme of the approach used in Chapters 3 to 6 to investigate the toxicity of ZnO, Ag and CeO_2 NPs to the terrestrial isopod *Porcellionides pruinosus*. Comparative forms included ionic ($ZnCl_2$ and $AgNO_3$) and non-nanosized ZnO forms, as well as the combined exposure of CeO_2 NPs and phenanthrene.

1.5 Relevance of the dissertation

Metal-based nanoparticles present unique characteristics that distinguish them from the ionic counterparts and non-nano sized particles. As a consequence, they have been used in a diverse array of applications. One of the main applications of NPs is in consumer products that are used on a daily basis worldwide. Most NPs, especially the ones used in consumer products, end up in the wastewater treatment system. As described above, NPs are likely to end up in the sewage sludge in the wastewater treatment plants, and be discarded into the environment, potentially contaminating the soil through the land application of biosolids.

Initially, ecotoxicological studies were focused on the aquatic compartment and their organisms. However, soils were found to be an important compartment for environmental exposure to NPs, as transference from the aquatic compartment to soils will occur (Gottschalk et al., 2015; Gottschalk et al., 2009; Mueller and Nowack, 2008).

Most studies have evaluated the effects of metal-based NPs to soil invertebrates (e.g., nematodes, enchytraeids, earthworms, springtails), often using non-natural

media such as aqueous solutions, agar or artificial soils (Amorim and Scott-Fordsmand, 2012; Arnold et al., 2013; Gomes et al., 2013; Hu et al., 2010; Manzo et al., 2011; Starnes et al., 2015). To date, there is a lack of ecotoxicological studies on NPs carried out in natural soils. Thus, assessing the effects of metal-based NPs to soil organisms is a relevant issue. Terrestrial isopods are known to play an important role in soil functioning, and are suitable for assessing the effects of metals in soil. Nevertheless, only few studies were performed on the toxicity of NPs to isopods in soil, with the few studies available often only using dietary exposure (Drobne et al., 2009; Novak et al., 2012; Pipan-Tkalec et al., 2010; Valant et al., 2012). Therefore this thesis evaluates the toxicity of three metal-based NPs to the isopod *Porcellionides pruinosus*, using exposures in natural soil and also comparing soil exposure with dietary exposures.

The results from this thesis were already used within a report requested by ECHA-the European Chemicals Agency, and can therefore be used also in the future to enhance the databases regarding nanoparticle hazard and risk assessment.

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CHAPTER 2: METAL-BASED NANOPARTICLES IN SOIL: FATE, BEHAVIOR, AND EFFECTS ON SOIL INVERTEBRATES

Paula S. Tourinho[1], Cornelis A. M. van Gestel[2], Stephen Lofts[3], Claus Svendsen[4], Amadeu M. V. M. Soares[1] and Susana Loureiro[1]

[1] Department of Biology & CESAM, University of Aveiro, 3810 193 Aveiro, Portugal

[2] Department of Ecological Science, Faculty of Earth and Life Sciences, VU University, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

[3] CEH Lancaster, Lancaster Environment Centre, Lancaster, LA1 4AP, United Kingdom

[4] Centre for Ecology and Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, Oxfordshire OX10 8BB, United Kingdom

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2.1 Abstract

Metal-based nanoparticles (NPs) (e.g., silver, zinc oxide, titanium dioxide, iron oxide) are being widely used in the nanotechnology industry. Because of the release of particles from NP-containing products, it is likely that NPs will enter the soil compartment, especially through land application of sewage sludge derived from wastewater treatment. This review presents an overview of the literature dealing with the fate and effects of metal-based NPs in soil. In the environment, the characteristics of NPs (e.g., size, shape, surface charge) and soil (e.g., pH, ionic strength, organic matter, and clay content) will affect physical and chemical processes, resulting in NP dissolution, agglomeration, and aggregation. The behavior of NPs in soil will control their mobility and their bioavailability to soil organisms. Consequently, exposure characterization in ecotoxicological studies should obtain as much information as possible about dissolution, agglomeration, and aggregation processes. Comparing existing studies is a challenging task, because no standards exist for toxicity tests with NPs. In many cases, the reporting of associated characterization data is sparse, or missing, making it impossible to interpret and explain observed differences in results among studies.

Keywords: metal; nano; particles; soil; toxicity; invertebrates

2.2 Introduction

Nanoparticles (NPs) are defined as particulate matter with at least one dimension less than 100 nm (Christian et al., 2008). Although materials of this size are naturally present in the environment, manufactured NPs may have distinctive surface properties and chemistry in comparison with natural NPs (Handy et al., 2008). Manufactured NPs have a large area-to-volume ratio and size-dependent properties, especially smaller particles (<20 nm), which infers novel properties and behaviors that make them suitable for nanotechnology applications (Auffan et al., 2009). The commercially useful properties of nano-sized materials have resulted in large-scale production of NPs and NP-containing products. The expanding range of applications for NPs has brought about a rapid development of the nanotechnology industry. The vast majority of NP types in current use are metal-based NPs, e.g., nanosilver, zinc oxide, titanium dioxide and iron oxide (Lawton, 2008).

While the size-dependent behavior observed in nanoparticles may be desirable and indeed essential in commercially produced materials, their implications for the fate and effects of NPs in the environment need to be considered. It is thus essential to understand as far as possible the basic physicochemical properties of NPs and to relate these to fate and toxicity in order to predict effects under environmental conditions.

The number of studies regarding the effects of NPs is increasing rapidly, especially for the aquatic environment. A number of reviews have considered the behavior and effects of NPs in water (Fabrega et al., 2011; Klaine et al., 2008; Moore, 2006; Nowack and Bucheli, 2007; Scown et al., 2010). However, when it comes to the soil compartment or aquatic sediments, relatively little information has been generated to date. In order to close this gap in the literature, this review aims to summarize the available data and theories related to the factors affecting the behavior, fate and bioavailability of manufactured metal-based NPs in soils, and their toxicity to soil organisms. Soil invertebrates play an important role in soil ecosystem function (e.g., decomposition and nutrient recycling) and thus addressing NP effects on these organisms is crucial to understand the potential impacts of NPs in the soil environment.

2.3 Sources of metal-based nanoparticles in soil

The physics and nanotechnology behind current metal-based NPs were developed decades ago, with products reaching the marketplace some years ago and new commercial applications for nanostructures being developed continuously (Wardak et al., 2008). Metal and metal oxide NPs have been introduced in many products with many different purposes. For example, they have been used in consumer products such as sunscreens and cosmetics (ZnO and TiO_2) (Mu and Sprando, 2010; Newman et al., 2009), detergents and antibacterials (Ag) (Amendola et al., 2007; Baker et al., 2005; Benn and Westerhoff, 2008; Duran et al., 2007; Panáček et al., 2006), paints (TiO_2 and Ag) (Kaegi et al., 2010; Kaegi et al., 2008), printer inks (Ag and Au) (Cui et al., 2010), and textiles (Ag) (Benn and Westerhoff, 2008; Zhang et al., 2009). The applications of nanoparticles also include electronics, medicine, chemistry, catalysis, and fuel additives (Morris and Willis, 2007).

Due to the diversity of nanotechnology applications, nanoparticles may enter the environment through many pathways. The sources of NPs to the environment are complex, consisting of both point and diffuse releases. During industrial production and transportation, accidental spills may occur (Navarro et al., 2008). Emissions to the atmosphere may result in deposition to soils and waters from various sources (e.g., waste incineration). Further deliberate additions to the environment may occur via the use of NPs in soil and water remediation technologies (Barnes et al., 2010) and agriculture (e.g. as fertilisers). The major source of NP deposition onto land is currently through the disposal of WWTP sewage sludge where NPs that are released from consumer products into wastewaters may partition into sewage sludge during the wastewater treatment process (Mueller and Nowack, 2008). For example, Ag NPs can be released during the washing of textile products, in quantities that vary depending on the compounds used in the washing and on how the particles were incorporated in the textile (Benn and Westerhoff, 2008; Geranio et al., 2009). Titanium dioxide used as a white pigment can be released from paints (Kaegi et al., 2008). This release of metal-based NPs into domestic and industrial wastewaters upstream of treatment is believed to result in the most significant input of NPs into the environment, via treated wastewater effluent and sewage sludge (Gottschalk and Nowack, 2011). Experimental data shows that up

to 99% of TiO_2 NPs entering wastewater treatment plants (WWTPs) are retained within the sludge phase, thus ending up in the terrestrial rather than the aquatic environment (Johnson et al., 2011). The main input of NPs to soils is believed to be due to land application of sewage sludge (Gottschalk et al., 2009; Johnson et al., 2011; Mueller and Nowack, 2008). It is, however, unlikely that NPs will enter the soil in their original form due to the organically rich and reactive environments of WWTPs (Gallego-Urrea et al., 2010). Silver sulfide nanoparticles, found in sewage sludge, are suggested to have been formed from the reaction of Ag NPs with sulfide in a reductive environment (Kim et al., 2010).

Despite our knowledge of potential environmental release pathways, little information is available about the quantities of NPs currently produced, let alone those reaching the environment (Gottschalk et al., 2009; Johnson et al., 2011). Some authors have estimated expected levels of NPs in the environment using various fate models. For example, it has been estimated that the use of sewage sludge could contribute to inputs of 1 $\mu g/kg^3$ and 120 $\mu g/kg^3$ of Ag and TiO_2 NPs to agricultural land per year, respectively (Mueller and Nowack, 2008). Regarding Ag, logically the quantity of NPs produced will be the main factor determining the concentrations found in all environmental compartments, as natural background levels of Ag can be considered very low (Gottschalk et al., 2009; Johnson et al., 2011). Others metals, however, occur naturally in greater concentrations. For example, abundances of TiO_2 in a dataset of 845 topsoils from across Europe ranged from 0.02% to 5.5% (Salminen et al., 2005).

While accurate production estimates are currently difficult to make, it is suggested that TiO_2 and ZnO NPs would be the most common particles in the environment due to their large-scale production (Gottschalk et al., 2009; Johnson et al., 2011).

Other factors likely to determine environmental concentrations of NPs include the formulation of NP-containing products, the properties of the NPs, and spatial patterns of use, the latter being largely controlled by spatial variability in population density (Gottschalk et al., 2009; Johnson et al., 2011).

2.4 Behavior of metal-based nanoparticles in soil

Manufactured metal-based NPs possess a number of key characteristics that are believed to exert important controls on their environmental behavior, fate and ecotoxicity. These include physical characteristics, particularly size and shape, and chemical characteristics such as the acid-base character of the surface and the aqueous solubility of the metal. These characteristics in turn will determine the extent to which metal-based NPs undergo transformations that will control their fate, behavior and ecotoxicity in the environment. Such processes will include (but not necessarily be limited to) aggregation/agglomeration, sorption to surfaces, and dissolution to the ionic metal. Furthermore, metal–based NPs are frequently manufactured with surface coatings, which may modify their intrinsic behaviour. The existence of these multiple characteristics implies that characterisation of NP form and presentation in the environment will be key to understanding their behaviour, fate and ecotoxicity. Soil represents a relatively complex medium for the understanding of the physicochemical behavior of manufactured NPs. In comparison to the dissolved phase, where behaviour can be understood largely in terms of particle stability against aggregation, soils present a solid matrix with which NPs may interact, as well as an aqueous phase which may contain appreciable amounts of natural colloidal/particulate material. In the context of ecotoxicity, a key issue is the understanding of how specific organisms are exposed to NPs present in different phases (soil, soil water) and how the presentation of the NPs within these phases further influences exposure. The effective exposure level of organisms may not be assessable by elemental mass concentrations alone and additional information on the presentation of the NPs is likely to be essential for understanding and predicting their effects. Since much of the existing work on the behaviour of NPs has been done in aqueous media, we will include examples of such work in this section. Such work is itself directly relevant to soils since NP behaviour in the aqueous phase of soils may be of considerable importance for their transport and bioavailability. Furthermore, patterns of NP behaviour in the aqueous phase may provide useful information regarding their presentation in the solid matrix itself.

The assessment of the form and presentation of NPs in environmental matrices, and soils especially, is currently hampered by the relative lack of appropriate procedures for their characterisation, and development of suitable techniques for better characterisation poses a great challenge (Hassellöv et al., 2008). For relatively simple analyses, such as the aggregation state of NPs in aqueous solution, it is possible to use dynamic light scattering (DLS) or microscopy-based techniques such as scanning and transmission electron microscopy (SEM and TEM) and atomic force microscopy (AFM) (Tiede et al., 2009). However, quantifying the state of NPs in soils, compared to solutions, is hampered by the need for knowledge of the NP presentation within the soil matrix, rather than in aqueous extracts of the soil. Currently most characterisation techniques are limited by only being applicable in the aqueous phase (Tiede et al., 2009).

Stone et al. (2010) have suggested that the main NP properties that should be evaluated in ecotoxicological studies to describe exposure are dispersability, agglomeration/aggregation, dissolution rate, size, surface area and charge, and surface chemistry. These properties are likely to be key in controlling NP stability, and consequently on their transport through the environment and availability to organisms. Therefore, quantification of these properties, and of how they are modified by NP interactions with soils, will enable more accurate assessment of which NP properties influence bioavailability and toxicity across soil types, concentration ranges and timeframes.

Aggregation and agglomeration

Aggregation is defined as the association of primary particles by strong bonding, while agglomeration is defined as association by weak bonding caused by Van der Waals forces (Jiang et al., 2009). However, in both papers on water and soil exposure studies with NPs the terminology for aggregates and aggregation is often used in cases where possibly only agglomeration has occurred (i.e., where there is no actual permanent sintering of the particles). In the remainder of this review, we have followed the terminology used by the authors of the original papers, but attention should be given to the correct use of terminology in future

work, as this will be important for evaluating size-specific effects in terms of toxicity.

In the environment, physical forces (e.g., Brownian motion, gravity, and fluid motion) and NP characteristics (e.g., surface properties, particle size) will affect NP agglomeration and aggregation (Farré et al., 2009). The particles are constantly colliding with each other due to Brownian motion, and agglomeration will occur when the energy of either motion or attraction exceeds the energy of repulsion (Lin et al., 2008). To aggregate, the cores of particles must make contact; thus, the aggregation rate is believed to be proportional to the probability of collision between two particles (Rosická and Šembera, 2011). Aggregation may result in the formation of particle flocks of sufficient size to sediment out of a solution gravimetrically.

It has also been shown that the NP aggregate size in solution depends on properties such as initial particle size and concentration (Figure 2.1). Using iron nanoparticles, Phenrat et al. (2006) found that higher concentrations (60 mg/L) resulted in higher aggregation rates and stability of aggregate size in comparison to lower concentrations (2 mg/L). Zinc oxide NPs dispersed in aqueous solution aggregated in a wide range of sizes, resulting in aggregates almost 10-fold larger than the primary NPs (Pipan-Tkalec et al., 2010; Wang et al., 2009). Nevertheless, not all particles were incorporated into aggregates and individual NPs were also detected in suspensions (Lin and Xing, 2008; Wang et al., 2009). Thus, the size distribution of aggregates may vary among particle types. For example, TiO_2 NPs showed a uniform distribution and agglomeration (Jemec et al., 2008), but ZnO NPs showed a wide distribution size and aggregation (Pipan-Tkalec et al., 2010).

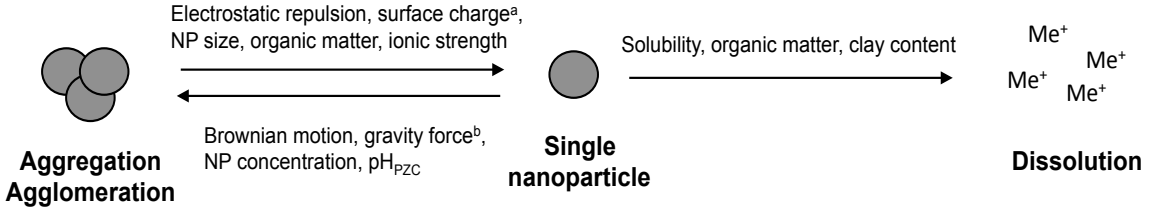

Figure 2.1: Factors affecting the processes of aggregation/agglomeration and dissolution of single nanoparticles in the environment. [a] Considering similar surface charge. [b] Acting only on larger particles.

Surface coating

The chemistry of the medium will influence the electrostatic surface charge of the particles thereby affecting agglomeration/aggregation rates and particle stability (Figure 2.1). In the absence of a surface coating, metal-based NPs have charged surfaces resulting from the presence of hydroxyl (–OH) groups that can take up and release protons, and can take up dissolved chemical species such as metal ions and ligands. The sign and magnitude of the surface charge will be determined by the intrinsic chemistry of the surface groups and the chemical composition of the solution, in particular the pH and the concentrations of binding species. Surface charging results in the formation of an electrical double layer (EDL), comprising the charged surface sites and a diffuse layer containing ions attracted from the solution to the particle surface in response to the charge. The electrical potential at the interface of the diffuse layer and the bulk solution (the zeta potential) can be measured, and its variation with solution chemistry can effectively be used as a surrogate for the variation in particle surface charge with solution chemistry. The zeta potential of uncoated metal or metal oxide particles typically decreases from positive values at low pH to negative values at high pH. As the pH approaches the iso-electric point (IEP), or the "point of zero charge", where particle charge/zeta potential approaches zero, the aggregation rate will increase (Dunphy Guzman et al., 2006; Jiang et al., 2009) due to the lowering of the electrostatic repulsive forces between particles. It is considered that a suspension of homogenously charged particles will be stable when the magnitude of the zeta potential is greater than 30 mV. Jiang et al. (2009) found that the hydrodynamic diameter of TiO_2 NP aggregates varied with the solution pH, being greatest at the IEP (pH 6.0). Fabrega et al. (2009) reported that Ag NPs stabilized by citrate coating showed no variation in aggregate size over the pH range 6 to 9, consistent with the findings of El Badawy et al. (2010) for similarly stabilized Ag NPs. Here, the invariant aggregate size may be explained by the relatively small variation in zeta potential of the particles in this pH range (El Badawy et al., 2010). It was also observed that small aggregates of Ag NPs adsorbed humic acid and that this adsorption resulted in disaggregation (Ed. disagglomeration) of the NPs (Fabrega et al., 2009).

The presence of a surface coating on manufactured NPs may significantly modify their surface chemistry, compared to the uncoated equivalent. For example, El Badawy et al. (2010) measured contrasting surface charging behaviour of different types of Ag NP, one uncoated and the remainder coated with substances imparting contrasting surface chemistries. Under environmental conditions, the stability of such coatings over time will be important in determining for how long the particles maintain the manufactured surface properties. Properties influencing coating stability will include the reversibility of the coating process and the (bio)degradability of the coating over time.

Classical colloid theory can be applied to metal and metal oxide NPs to help explain their stability. The Derjaguin, Landau, Verwey and Overbeek (DLVO) theory considers stability as a function of the repulsive (i.e. electrostatic) and attractive forces (i.e., Van der Waals) to which a particle is subjected (Ben-Moshe et al., 2010). Although DLVO theory has generally proved unsatisfactory for the quantitative prediction of colloid behavior in complex natural environments, the conceptual framework that it provides for considering such behavior can be useful in explaining trends in observed behaviour in such systems. Some applications of the theory have been made to explain the stability of manufactured NPs in aqueous media (e.g., Buettner et al., (2010), Ju-Nam and Lead, (2008), Stebounova et al., (2011)).

Dissolution

Some types of metal-based NPs are thermodynamically unstable and undergo chemical dissolution if kinetic restrictions (e.g., a non water-soluble surface coating) are absent. Dissolution occurs when an ion detaches from the particle and migrates through the EDL into the solution (Borm et al., 2006). The dissolution of metal-based NPs releases ionic species that may themselves be toxic. Thus, the extent of dissolution and the relative toxicities of both the nanoparticulated and dissolved forms need to be considered to better understand the potential nanoparticle effects on organisms over time.

The dissolution behaviour of non-nanosized metals and metal oxides in soils may therefore be of use in prioritizing NPs for study and in choosing which soil

properties to consider that may influence their dissolution rates. For example, a non-nanosized ZnO material was shown to dissolve almost completely in soils over nine months, with concomitant formation of a number of zinc precipitates (Milani et al, 2010). However, the number of studies on NP dissolution in soil remains very small due to the technically challenging nature of such work. In soils, it was found that Au NPs of different sizes (20 and 55 nm) showed 8.0 and 3.4% dissolution of Au ions after 28 d, respectively (Unrine et al., 2010b). About 10 to 17% of Ag NPs (30-50 nm) were found to be transformed to Ag(I) after 28 d, suggesting oxidative dissolution to ions (Shoults-Wilson et al., 2011b). The dissolution of CeO_2 NPs has been shown to be very low in different types of soil (Cornelis et al., 2011). The dissolution (Ed. dispersion) of Ag and CeO_2 NPs has been shown to vary with soil composition (Cornelis et al., 2010). These authors concluded that dissolved Ag tended to bind to organic matter or clay present in soils, with the sorption of Ag NPs increasing with increasing clay content of the test soils (Cornelis et al., 2010). The solubility of ZnO nanoparticles has been shown to be very similar in comparison to non nanosized ZnO of <1 μm (Milani et al., 2010) and >200 nm (Kool et al., 2011; Milani et al., 2010). More studies are needed on the longer term dissolution of metal-based NPs in order to understand better the fate of such NPs in soils.

Soil properties

Nanoparticle behaviour within soil systems will be further complicated by the presence of the solid phase. Soil components such as clay particles or humic molecules will themselves have charged surfaces, which will influence the association of NPs with the solid phase. Such soil components may also form colloids in the aqueous phase, which will interact with NPs. For example, humic molecules desorbing into the aqueous phase may sorb to NP surfaces, and so influence NP stability. All these processes are themselves strongly influenced by overriding characteristics of the soil system, particularly the pH and the ionic strength of the aqueous phase. Currently, work on the behavior of metal-based NPs in soils has focused on soil suspensions rather than intact soils. For example, Gimbert and co-workers (2007) studied the particle size distribution of ZnO NPs in

<1 μm size suspensions extracted from a high pH soil 0,7, and 14 days following spiking. The NPs were found to quickly equilibrate between the aqueous and solid phases and concentrations in the <1 μm fraction were stable during the experimental period.

The aggregation rate of TiO_2 NPs in soil suspensions has been found to be negatively correlated to soil characteristics such as dissolved organic matter and clay contents, and positively correlated to the ionic strength, zeta potential, and pH (Fang et al., 2009). Nanoparticles sorbed less strongly to soils of low ionic strength and high dissolved organic matter content, suggesting these factors may affect the bioavailability of metal-based NPs in soils, assuming that bioavailability is related to the particle fraction suspended in the pore water and not the fraction associated to the soil matrix. Other studies showed that aggregation of TiO_2 NPs occurred at a ionic strength higher than 4.5 mM (French et al., 2009) and that increasing ionic strength from 1 to 100 mM caused a 50-fold increase in TiO_2 NP diameter (Jiang et al., 2009).

The ionic strength of the medium affects the stability of the diffuse layer in the EDL. An increase in the ionic strength will lead to a decrease in the EDL thickness, which favours particle association and leads to increasing agglomeration. The effects of ionic strength on the aggregation of Ag NPs (uncoated, citrate, and sodium borohydride-coated particles) were observed for suspensions with pH higher than 7 (El Badawy et al., 2010). This demonstrates that here the particles were stabilized by electrostatic repulsion, caused by the predominance of negative forms (i.e., anions) in the medium and the negative charge of the particles. In contrast, the agglomeration of Ag NPs coated with polyvinylpyrrolidine (PVP) was not influenced by increasing ionic strength (El Badawy et al., 2010). This reflects that here the particles were stabilised due to steric repulsions caused by the uncharged coating material, the effectiveness of which was not influenced by ionic strength.

Overall, the stability and sorptive behaviour of metal-based NPs in soils is likely to be of importance for transport, fate and toxicity, yet current studies have focused on behaviour at relatively short timescales and there has been a focus on investigations at soil:solution ratios that are low compared to both ecotoxicity

testing and field conditions, and at relatively short timescales. It is currently unclear whether the outcomes of such investigations can be robustly applied to higher soil:solution ratios and longer, more environmentally relevant timescales. More research is needed on NP behaviour in intact soils and over timescales of months to years.

In soils, dissolved or particulate organic matter can sorb to NP surfaces. This sorption may influence particle properties in a number of ways. Humic substances are negatively charged at environmental pHs and thus their sorption will make the overall particle-humic conglomerate negatively charged (Ghosh et al., 2008). This may increase particle stability in solution, reducing aggregation and settling (Ben-Moshe et al., 2010; Fang et al., 2009). The alteration of the surface charge may also decrease particle affinity for cell membranes and thus reduce their bioavailability and uptake (Unrine et al., 2008). Steric hindrance effects may also contribute to the enhanced stability of humic-coated nanoparticles. On the other hand, Ghosh and co-workers (2008) showed that at low pH, humic acid (HA) caused aggregation of Al_2O_3 NPs. Here, the charge of the HA appeared sufficiently low to allow its aggregation due to hydrophobic interactions; thus, HA-coated particles also became susceptible to aggregation. Kool et al. (2011) presented TEM images showing ZnO NPs bound to solid phase organic matter in a soil with a pH of 5.5, suggesting that under suitable conditions organic matter may destabilize particle dispersions. The overall effect of humic substance sorption on particle stability and bioavailability appears to be a complex function of factors, particularly the soil pH and the intrinsic hydrophobicity of the humic substances.

Because of the limitations of analyzing NPs in the soil matrix, most studies characterize the particles in their pristine form prior to addition to the soil, or in the aqueous solution used to contaminate the soil. In these studies, the particles are typically found to be agglomerated/aggregated, for example TiO_2 (French et al., 2009; Keller et al., 2010; Lecoanet et al., 2004; Zhu et al., 2009), ZnO (Franklin et al., 2007; Hooper et al., 2011; Keller et al., 2010; Lin and Xing, 2008; Zhu et al., 2009), Ag (Asharani et al., 2008; Navarro et al., 2008), SiO_2 (Canesi et al., 2010), and CeO_2 (Keller et al., 2010). Manzo et al. (2010) analyzed a ZnO NP

contaminated soil by the Brunauer–Emmett–Teller (BET) method and found that the particles were not aggregated. The author attributed this result to the spiking procedure, which consisted of mixing a dry powder of the NPs with dry soil. However, the solution extracted from soil samples wetted after spiking showed larger particles, where a bimodal peak could be observed in a DLS analysis ranging from 103 to 470 nm. Therefore some attention should be given to this issue when designing experiments and choosing the spiking procedures. Although the review by Handy et al. (2012), on practical experiences and recommendations, does mention soil, only little attention has been given to soil experiments. But studies with NPs in soils already performed have presented different contamination methodologies including mixing the NPs powder directly in with the soil, adding a stock dispersion made in distilled water to soil (e.g., Unrine et al., (2010a)) or preparing a stock dispersion in soil elutriate that is then mixed in with the soil (e.g., Kool et al. (2011)). There is a general need to develop standard procedures for soil dosing with NPs in order to improve the comparability of multiple test results. Regarding soil variability, Handy et al. (2012) recommended the use of an artificial soil as a benchmark for exposure evaluation of NPs. This is a useful recommendation in order to compare the behaviour and toxicity of different types of NPs in a consistent soil medium. For better understanding of nanoparticle behaviour as a function of soil properties, however, studies on multiple soils are additionally needed.

2.5 Transport of metal-based nanoparticles in soil

The interaction occurring between particles and solid surfaces will control the transport of NPs through soils (Darlington et al., 2009). This interaction may be influenced by both environmental conditions and the physicochemical characteristics of the particles (Figure 2.2). Generally, those factors that influence the stability of NPs will also tend to influence transport properties. As has been established for colloids, the sedimentation and diffusion of NPs are caused by gravity and Brownian motion, respectively (Dunphy Guzman et al., 2006). In porous media, the transport of NPs is controlled by Brownian motion (Lecoanet et al., 2004). However, as the particles agglomerate and aggregate, gravitational

forces increase and the particles are more likely to interact with the surfaces of soil particles (Dunphy Guzman et al., 2006). The interaction between metal-based NPs and soil surfaces is also dependent on the surface charge of the NPs and the soil, since these factors influence aggregation and electrostatic attraction/repulsion among particles and between particles and soil. When the particle and the soil both have similarly charged surfaces, the repulsion between them will favour particle mobility as opposed to sorption. Where the particles and the soil have opposite charges, particles will be attracted to the soil and sorption may occur, thus decreasing mobility (Darlington et al., 2009). While specific functional coatings may be applied to metal-based NPs during manufacturing, sorption of ions such as humic substances may occur in the environment, potentially involving partial or complete replacement of any manufactured coating. Positively-charged Al_2O_3 NPs (50, 80 and 120 nm) demonstrated relatively low mobility (Darlington et al., 2009) due to sorption to negatively-charged soil surfaces. On the other hand, Al_2O_3 NPs (50 nm) with adsorbed phosphate, producing a net negative charge, showed reduced sorption to soil and hence higher mobility (Darlington et al., 2009). A similar effect of phosphate on CeO_2 NPs has also been noted (Cornelis et al., 2011). The ionic strength of the soil solution also influences the tendency of particles to sorb to the soil. Higher ionic strength decreases the repulsive forces between particles and between particles and the soil surfaces, in accordance with DLVO theory (Fang et al., 2009), leading to increased aggregation and sorption. For example, Ben-Moshe et al. (2010) showed that addition of sodium chlorate reduced the electrostatic repulsion between particles (Fe_2O_3, TiO_2, CuO, and ZnO NPs), resulting in aggregation and, thus, reduced mobility in a glass bead column. Fang and co-workers showed that high ionic strength reduced the transport of negatively charged TiO_2 NPs through soil columns (Fang et al., 2009).

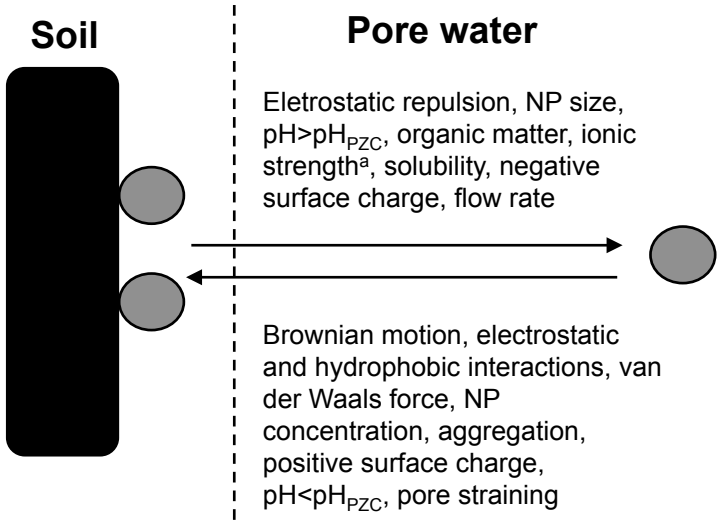

Figure 2.2: Factors affecting the partitioning of nanoparticles between soil and pore water. [a] Not valid for high values of ionic strength; dashed line is the diffusion layer. pH_{PZC} =point of zero charge.

Trapping of particles within soil pores (pore straining) is a physical mechanism influencing particle retention in soils. The importance of pore straining as a retention mechanism depends on the particle size and pore size distribution (Pennell et al., 2008). In general, the mobility of NPs in soil decreases with increasing particle size (Darlington et al., 2009), since larger particles are more likely to be retained by pore straining. Aggregation tends to increase the importance of pore straining as retention mechanism by producing a range of aggregate sizes that overlaps to a greater extent with the size distribution of soil pores. The influence of pore straining in particle retention thus tends to depend upon similar factors to those controlling aggregation, for example pH and ionic strength (Ghosh et al., 2008). Increases in particle size due to aggregation may also be associated with chemical conditions favouring sorption to the soil (e.g., pH ~ IEP). While smaller particles are more mobile and are more likely to penetrate to groundwater depth (Darlington et al., 2009), larger aggregates tend to be retained in the upper layers of soils, potentially causing soil clogging (Fang et al., 2009). Under laboratory conditions, particle transport has also been shown to be modified by the accumulation of particles in the soil and consequent modification of the effective pore size distribution (Darlington et al., 2009), although it is unclear to what extent this process may be important under field conditions. The latter authors found that concentration and the low solubility of Al nanoparticles were

important factors in this process (Darlington et al., 2009). However, when testing a sandy column, the mobility of Al_2O_3 was larger than in soil, showing the importance of the matrix constitution on the transport of NPs (Darlington et al., 2009).

The solution flow rate has also been shown to affect the mobility of particles. When the flow rate is reduced, the mobility of NPs was found to be smaller (Ben-Moshe et al., 2010; Jeong and Kim, 2009). Flow rate was found to affect not only the deposition of CuO NPs, but also their aggregation in a porous medium (Jeong and Kim, 2009).

2.6 Toxicity and bioaccumulation of metal-based nanoparticles in soil invertebrates

Ecotoxicological tests to determine the toxicity of NPs to soil organisms can be conducted with different exposure media (e.g., soil, food, and water) (Table 2-1). Also, comparison of the toxicity with that of corresponding metal salts or non-nanosized material is essential to determine to what extent the toxicity is caused by ions formed by NP dissolution or directly related to the nanosize of the particles.

When applying already standardized or well described protocols to test NP toxicity in soils, some adaptations may have to be needed to ensure robust dosing and exposure of the organisms. Also when looking at the effects of NPs and comparing them with their non-nano counterparts, different modes of action and therefore different effects may be expected. Therefore the selection of an endpoint should be considered a key factor when comparing toxicities of NPs and their non-nano counterparts, and such comparisons need careful discussion. In addition, some tests apply aqueous exposure (e.g., nematodes and earthworms), which seem to be quite unrealistic compared to conditions in soil. Such tests however, have provided new insights that have helped to understand NPs behaviour in standardised (or well established) media previously used for metal exposure. Ecotoxicity testing using aqueous media instead of soil, such as the *Caenorhabditis elegans* test in liquid medium, or hydroponic plant tests, may avoid the current technical difficulties for the characterization of NP exposure in soil. But

of course, conclusions from such tests must take into account the likely differences between NP behaviour in water/hydroponic solutions and soil.

The process of bioaccumulation of metals in soil organisms and biomagnification through the trophic chain is well known in the literature. For metal-based NPs, bioaccumulation has been evaluated in a few studies, while biomagnification has scarcely been addressed. The bioaccumulation and biomagnification of Au NPs of three different sizes were evaluated in hornworms via food consumption of tobacco leaves (Judy et al., 2011). The authors found that the biomagnification was related to nanosized Au and not to dissolved ions. This process was related to zeta potential rather than particle size, since higher biomagnification was found for particles having the same zeta potential but with different sizes (10 and 15 nm) (Judy et al., 2011).

Some metal-based NPs (e.g., TiO_2) are likely to be persistent in the environment due to their very low to negligible dissolution, so it is likely that they will accumulate in soil and water compartments (Baun et al., 2008). Indeed, NPs will dissolve partly, and form aggregates and agglomerates. Kinetics experiments have shown TiO_2 NPs to be very stable after forming aggregates in water and soil solution (French et al., 2009). For such long term stable NPs bioaccumulation may occur as, it is believed that NPs may be readily taken up if present in the soil pore water (Unrine et al., 2008). Biomagnification may also occur, once the NPs interact with organic molecules inside an organism (e.g., lipids and carbohydrates) and then become more available for trophic transfer (Unrine et al., 2008).

Direct (dermal) uptake via the skin is also a possible route of exposure in soil organisms. The negatively charged cuticle of nematodes, for example, has been shown to attract NPs, to a greater degree than a comparable bulk material (Wang et al., 2009). However, the major route of exposure may be ingestion of contaminated particles or contaminated food (Hu et al., 2010). ZnO and TiO_2 NPs were both accumulated in the tissues of earthworms, with ZnO showing greater accumulation than TiO_2 (Hu et al., 2010). The authors suggest that the properties of ZnO nanoparticles result in a greater bioavailability.

Overall, the importance of the exposure route will be, as for other chemicals, species specific. For example, collembolans will mainly be exposed to soil pore

water, earthworms to both pore water and soil particles by dermal and oral contact, and woodlice to food (decaying leaf material) and soil particles by ingestion and to a limited extent to soil pore water.

Silver

Silver nanoparticles have been used mainly as bactericides in products, and are believed to induce toxicity through a combination of effects from both dissolved ions and particles. Silver NPs cause the formation of reactive oxygen species (ROS) in the cells, though their toxicity also involves mechanisms than ROS (Choi and Hu, 2008). A recent study showed that the nematode *C. elegans* had increased ROS formation when exposed to 1 mg/L of Ag NPs (20-30 nm in K-media) while no increase in ROS production was seen from the matching AgNO3 exposure (Lim et al., 2012). This confirms indications from previous studies in *C. elegans* by the same authors where Ag NPs caused reproductive toxicity, which was related to oxidative stress, while no effects were seen on survival and growth (14~20 nm Ag NPs up to 0.5 mg/L in K-media) (Roh et al., 2009). While liquid K-media has obvious practical advantages some aggregation will occur (due to high ionic strength) and prevent effective high concentration exposures. Dosing citrate-coated Ag NPs into nematode growth media agar (NGM) produced a greater Ag NP stability and a more homogeneous distribution than dosing in K-medium significant aggregation was observed (Kim et al., 2012). Direct dose comparison with K-media studies are of course not possible, but using NGM the authors found an LC50 of 55 mg/L with mortality increasing to 60% at 100 mg/L, and that reproduction were reduced by >30% at 5 mg/L decreasing further to a 40% reduction at 100 mg/L, with neither endpoint showing any further increase in effect above 100 mg /L. The exposed *C. elegans* showed epidermal damage at 10 mg/L and high-resolution microscopy images showed NPs present in the cells of the digestive organ, but TEM-EDS analysis did not detect the presence of Ag, although osmium and uranium, used in pre-treatment of the NPs, were identified. Moreover, the effects of Ag NPs on survival and reproduction of *C. elegans* were not related to Ag+ concentration, indicating that the toxicity was mainly caused by Ag NPs acting directly on the surface of the nematode without NPs being taken up

(Kim et al., 2012). This series of studies show how non-soil media can contribute to understanding of the basic mechanisms involved in NP toxicity.

In the earthworm *Eisenia fetida*, Ag NPs caused total reproductive failure effects at 1000 mg/kg dry soil (Heckmann et al., 2011). The Ag NPs had negative zeta potential and were stable in suspension (Heckmann et al., 2011). These results are in accordance with the conclusion that the negative zeta potential of NPs causes repulsion to the soil, leading to a greater bioavailability.

Although size influences the toxicity, soil type was found to be more important than particle size (Shoults-Wilson et al., 2011b). In a sandy loam, the earthworm *E. fetida* accumulated significantly more Ag from Ag NPs exposure than in artificial soil. Sandy loam characteristics, such as lower pH and organic content, resulted in greater availability of Ag ions (Shoults-Wilson et al., 2011b). Also, a natural sandy loam resulted in greater avoidance of Ag NPs by earthworms in comparison to artificial soil (Shoults-Wilson et al., 2011a). Even though no difference in avoidance behavior was observed for particles having different size or coating material, smaller particles (15-25 nm) coated in citrate were avoided more than larger (30-50 nm) PVP-coated Ag NPs (Shoults-Wilson et al., 2011a). Avoidance behavior was found to be more sensitive than mortality, growth, and reproduction (Shoults-Wilson et al., 2011a).

Silver nitrate had greater reproductive toxicity than NPs for *E. fetida*, although no significant differences were found for effects on growth and survival (Shoults-Wilson et al., 2010). Not only the endpoint analyzed, but also the exposure time has great influence on the toxicity of NPs and metal salts. The avoidance rate for Ag NPs and AgNO3 was the same only at the end of the 48h avoidance test, although $AgNO_3$-contaminated soil was avoided immediately by the animals (Shoults-Wilson et al., 2011a). It was concluded, based on another study (Shoults-Wilson et al., 2011b), that the release of Ag ions from Ag NPs was not the major factor responsible for the avoidance effects, as the exposure time (48 h) would not be long enough to dissolve a similar amount of ions as presented in the AgNO3 based exposure (Shoults-Wilson et al., 2011a).

The accumulation of Ag seems to be greater in the earthworm *E. fetida* exposed to the corresponding $AgNO_3$ salt than from NPs (Heckmann et al., 2011; Shoults-

Wilson et al., 2011b). However, extended X-ray absorption fine structure spectroscopy (EXAFS) analysis in soil showed that silver in earthworms exposed to Ag NPs suggests accumulation was not only caused by the ionic form (i.e., ions dissolved from NPs), and that non-dissolved particles contributed to Ag accumulation in the organisms (Shoults-Wilson et al., 2010).

Aluminium

Only few studies have evaluated the toxic effects of aluminium NPs on terrestrial invertebrates. No mortality or reproductive effects (e.g., cocoon production, reproduction, cocoon hatchability) of Al_2O_3 NPs on *E. fetida* were observed at 1,000 mg/kg (Heckmann et al., 2011). Reproductive and behavioral effects of Al_2O_3 NPs on *E. fetida* were found only at concentrations as high as 3,000 mg/kg and 10,000 mg/kg field soil (pH 7.0, containing 1% of organic matter), respectively (Coleman et al., 2010). The authors observed no mortality caused by NPs, although aluminium body concentration was greater in the earthworms exposed to NPs than in those exposed to micro-sized particles. However, in comparison to $AlCl_3$, the Al_2O_3 NPs were less toxic for *C. elegans* tested in water media (Wang et al., 2009).

Gold

Effects on the reproduction of earthworms were comparable in Au NPs and Au ion ($HAuCl_4$) exposures, but no effects on earthworm growth or survival were observed from nanoparticles (Unrine et al., 2010a). Particles' size (20 and 55 nm) did not consistently affect NP accumulation in earthworms, where at the lower exposure concentration most Au was internalized from the 20 nm exposure, and at the highest exposure concentrations, higher body burdens arose from exposure to the 55 nm particles (Unrine et al., 2010a). The authors suggest that this may be due to a high level of aggregation of the 20 nm particles to >150 nm units in the higher exposure concentration while the 55 nm stayed mono-dispersed. When comparing the Au speciation in tissues from worms exposure to Au ions and NPs using μXANES, it was curiously found that both contained Au metal with no evidence of ionic forms (Au[I] and Au[III]) in the tissue. The spatial pattern of tissue

Au was more punctuated in the NP exposure than the ionic exposure suggesting Au NPs were taken up intact and remained so once internalised. This was corporate by the observation of induced metallothionein gene expression in the ion-exposed worms while there was no induction in the NP exposed worms. Furthermore, TEM analysis of the Au NP exposed worms showed electron dense areas in the gut epithelia matching the size of the respective NPs used, present either as single particles or groups of 2-7 particles within the cytoplasm. This study by Unrine et al. (2010a) is a very elegant demonstration of how important the use of multiple lines of evidence (physical, chemical, and biological) can be to draw the right conclusions.

Copper

Energy reserves (lipid, protein, and carbohydrate contents) of the enchytraeid *Enchytraeus albidus* decreased with increasing dose after 3 weeks of exposure to Cu NPs (80 nm) spiked at 100 and 200 mg Cu/kg in a field soil, but total energy reserve level was no longer affected after 6 weeks. Responses were similar for animals exposed to Cu NPs and $CuCl_2$ (Amorim et al., 2012). No mortality of *E. albidus* was observed upon exposure to Cu NPs (80 nm) at concentrations up to 1,000 mg/kg (Gomes et al., 2012). Both Cu NPs and $CuCl_2$ caused changes in gene expression, but responses were different, suggesting effects observed in NP exposed organisms therefore were attributed to the NPs and not only to Cu ions dissolved from Cu NPs, although some dissolution may have occurred as very low Cu^+ ion activity was measured in the soil solutions from both NP and ionic exposures (Gomes et al., 2012).

No effects were found on the survival, reproduction, growth, or gene expression of earthworms exposed to Cu NPs in artificial soil at concentrations up to 65 mg/kg dry soil (Unrine et al., 2010b). In this study, the corresponding salt ($CuSO_4$) was found to be more toxic than the NPs. Also, the animals exposed to smaller particles (between 20 and 40 nm) had greater Cu concentrations in the tissue and greater induction of methallothionein in comparison to animals exposed to larger particles <100 nm. However, a fraction of Cu NPs in the earthworms' tissue remained as NPs and did not release ions, so no relationship was found between

metallothionein gene expression and tissue concentration. Unrine et al. (Unrine et al., 2010b) concluded that Cu NPs may not cause subchronic toxicity to the same species in realistic conditions (i.e., < 65 mg/kg of Cu NPs) and that the uptake from soil is the route of entry for Cu NPs. Exposure of *E. fetida* to both Cu NP and $CuCl_2$, at 1,000 mg/kg in sandy loam soil, caused severe effects on reproduction, with effects being highest in the $CuCl_2$-exposed worms (Heckmann et al., 2011). The oxidation of NPs may lead to the release of Cu ions, which could be responsible for the toxicity. However, a drastic decrease in survival was only observed in the animals exposed to $CuCl_2$ (Heckmann et al., 2011).

Titanium dioxide

TiO_2 (50 nm) showed a 24-h LC50 for the nematode *C. elegans* of 80 mg/L (Wang et al., 2009). The toxicity of TiO_2 was found to be similar to aluminium oxide (LC50 = 82 mg/L), but less toxic than zinc oxide (LC50 = 2.2 mg/L) (Table 2-1).

Enzymatic activities of catalase (CAT) and glutathione S-transferase (GST) were not dose-dependently affected upon exposure of the isopod *Porcellio scaber* to food spiked with TiO2 NPs (Jemec et al., 2008). Also, no effects on feeding parameters (e.g., survival, feeding rate, food assimilation, and weight change) were observed in isopods exposed to 3,000 mg TiO_2 NP/kg dry food. Similarly, no effects of TiO_2 on survival, weight change, and GST were found for *P. scaber* also exposed to 1,000 mg TiO_2 NP/kg dry food (Drobne et al., 2009). However, CAT activity and feeding parameters were dependent on dose, exposure duration, NP size, and pre-treatment (sonicated and non-sonicated dispersions) (Drobne et al., 2009). The authors found that exposure duration was crucial in determining whether NPs were toxic, and the feeding parameters were increased by the presence of low doses TiO_2 NPs in a hormetic-like way.

Smaller TiO_2 NPs (7 nm) were found to be more toxic to the nematode *C. elegans* than greater particles (45 nm) (Roh et al., 2010). In comparison to non-nano sized, TiO_2 NPs were found to be more toxic for nematodes (Wang et al., 2009) and earthworms (Heckmann et al., 2011; Wang et al., 2009). However, both nano and non-nano TiO_2 significantly reduced the growth and reproduction success of *C. elegans* (Wang et al., 2009). Avoidance of *E. andrei* was observed at 1,000 (5 and

21 nm) and 5,000 mg TiO_2/kg artificial soil (10 nm), being related with TiO_2 specific surface area. An avoidance test conducted with TiO_2 NPs and micron-size (< 45 µm) showed a preference for micro-sized TiO_2 spiked soil only over the 5 nm TiO_2 NPs, which was not observed when testing 10 and 21 nm TiO_2 NPs (McShane et al., 2011).

For *E. fetida*, TiO_2 NPs caused reproductive toxicity at 1,000 mg/kg of dry natural soil (sandy loam), while micron-size TiO_2 (40 µm) did not affect reproduction (Heckmann et al., 2011). In addition, TiO_2 was the most toxic metal oxide NP for earthworms in comparison to Al_2O_3, SiO_2, and ZrO_2 (Heckmann et al., 2011). The earthworms *E. andrei* and *E. fetida* were exposed to 5, 10, and 21 nm sized TiO_2 NPs in artificial and field soil (McShane et al., 2011). In either soil, no effects on survival, growth, and reproduction were observed up to 10,000 mg TiO_2/kg soil. Reproduction (cocoon production) of earthworms decreased in artificial soil and sand with increasing TiO_2 concentration, but it was only significantly different from the control in the artificial soil test at 1,000 mg/kg (Cañas et al., 2011).

Bioaccumulation of TiO_2 NPs was not observed in the earthworm *Lumbricus terrestris* when exposed via food, soil, and water (Lapied et al., 2011). Although apoptosis could be observed in the most exposed tissues (e.g., cuticle and intestine), the NPs did not pass the tissue barrier, which prevented bioaccumulation (Lapied et al., 2011).

Zinc oxide

Zinc oxide is one of the most studied NPs in ecotoxicological tests with soil invertebrates. Genetic and physiological effects (CAT and superoxide dismutase activities or SOD) were observed in the earthworm *E. fetida* (Hu et al., 2010). The authors observed DNA damage and cellular damage at 1,000 and 5,000 mg/kg soil, respectively. CAT activity increased at the lower concentrations (100 and 500 mg/kg soil) and decreased at the highest concentration (5,000 mg/kg soil), in comparison to the control (Hu et al., 2010). SOD activity decreased significantly at concentrations above 500 mg/kg soil (Hu et al., 2010). Effects on survival, life history traits (change in weight and number of cocoons), and immune activity were compared in the earthworm *E. veneta* exposed to water, soil, and food spiked with

ZnO and $ZnCl_2$ (Hooper et al., 2011). Water-based exposure to ZnO NPs and $ZnCl_2$ showed comparable 24h LC50 values of 1.75 mg/L (95% confidence interval [CI] = 1.21 - 3.44) and 6.50 mg/L (95% CI = 0.69 - 15.95), respectively. In soil, the ZnO NPs reduced the earthworm reproduction by approximately 30% at 750 mg/kg, while a similar approximately 30% reduction in reproduction was seen at 250 mg/kg for $ZnCl_2$ and reproduction nearly ceased at 750 mg/kg for $ZnCl_2$. Similarly ZnO NP exposure did not affect the immune cell activity, while it was reduced to 70% at both 250 and 750 mg/kg for $ZnCl_2$. Although, the NPs were less toxic than $ZnCl_2$, the levels of zinc in the tissue were similar for the animals exposed to both ZnO NPs and $ZnCl_2$. This suggested that body Zn from ZnO exposure were internalized in a different and less active form, which was confirmed using backscattered electron imaging with accompanying EDX element maps showing inclusion of ZnO particles within the body wall tissues of the worms.
Effects of ZnO NPs on the survival of *E. fetida* were evaluated in sand and on filter paper (Cañas et al., 2011). Although high toxicity occurred in the filter paper test, it is a less realistic medium and causes greater stress to the organisms (Cañas et al., 2011). Additionally it should be considered that for ZnO, which is photo reactive, the filter paper test would also have added a potentially new mechanism of toxicity through effects of reactive oxygen species (ROS) generation from non-internalised ZnO, something that would not occur in the more realistic soil tests. The same authors also determined the effects of ZnO NPs on earthworm reproduction in sand and artificial soil. Reproduction showed a decrease with increasing NPs concentration in both tested media; however the values were not significantly different from control (Cañas et al., 2011).
The bioaccumulation of ZnO NPs in the isopod *P. scaber* was dose-dependent and was due to the dissolution of Zn, rather than the accumulation of ZnO particles (Pipan-Tkalec et al., 2010).
For the collembolan *Folsomia candida*, effects of ZnO NPs (<50 nm) on survival and reproduction were related to nanosize particles and not to the soluble form of zinc (ionic zinc) (Manzo et al., 2010). Conflicting results were found when testing ZnO (<100 nm) in a range of concentrations (Kool et al., 2011). Here effects on survival were not observed up to 6,400 mg/kg for ZnO NPs and non-nano ZnO

(200 nm), while $ZnCl_2$ showed a LC50 of 1,000 mg/kg. EC50 for reproduction was 1,964 (95% CI = 1635 - 2293), 1,591, and 298 (95% CI = 181 - 415) mg/kg for ZnO NPs, non-nano ZnO, and $ZnCl_2$, respectively (Kool et al., 2011). When the reproduction EC50 was based on Zn concentration in pore water it was similar for ZnO NPs and $ZnCl_2$ (10.1 and 16.8 mg/L, respectively), indicating that the toxicity of the NPs can be from dissolved ionic zinc (Kool et al., 2011).

The LC50 for effect on the survival of *C. elegans* was not significantly different between the zinc forms tested (bulk ZnO, $ZnCl_2$, and ZnO NPs) (Wang et al., 2009). Similarly, no significant difference in mortality was found between $ZnCl_2$ and ZnO NPs in a buffered K-medium (Darlington et al., 2009). However, when testing in unbuffered medium, higher mortality rates were observed for $ZnCl_2$, which was due to reduced complexation of Zn^+ ions with other species in the solution, in comparison to the buffered K-medium (Darlington et al., 2009). It is worthy to notice the great differences in LC50 found for *C. elegans* between these studies. Ma et al. (2009) found a mean LC50 of 789 mg Zn/L for ZnO, while Wang et al. (2009) reported an LC50 of 2.3 mg/L for ZnO, possibly due to differences in media used.

For effects on reproduction and avoidance behavior, no differences were found between $ZnCl_2$ and ZnO NPs, in neither medium tested (buffered K-medium and unbuffered media) (Darlington et al., 2009). However, induction of gene expression for metallothionein suggested that ZnO NPs were taken up by the cells or that dissociation into ions may enhance toxicity (Darlington et al., 2009).

Table 2-1: Toxicity of metal-based nanoparticles (NPs) to soil invertebrates; an overview of available literature data. See text for more details.

NPs	Initial size	Species tested	Concentration ranges	Exposure media	Duration	Endpoints	Outcomes	Reference
Ag	<100 nm	*Caenorhabditis elegans*	0.05 - 0.5 mg Ag/L	K-media	24h, and 72h (reproduction)	Survival, growth, reproduction		Roh et al. 2009
Ag	10 nm and 30-50 nm (PVP-coated)	*Eisenia fetida*	10 – 1,000 mg Ag/kg dry soil	Artificial and natural soil	28 d	Survival, growth, reproduction		Shoults-Wilson et al. 2011b
Ag	10 nm and 30-50 nm (PVP-coated)	*Eisenia fetida*	9.0-54 mg kg-1 (10 nm); and 0.3 - 27 mg kg-1 (30-50 nm)	Artificial and natural soil	2 d	Avoidance	EC50 = 8.7 mg/kg (10 nm) and 4.8 mg/kg (30-50 nm)	Shoults-Wilson et al. 2011a
Ag	30-50 nm (PVP-coated)	*Eisenia fetida*	1,000 mg/kg dry soil	Sandy loam soil	28 d	Survival		Heckmann et al. 2011
Ag	<100 nm	*Caenorhabditis elegans*	0.1 – 1 mg/L	K-media	24h, and 72h (reproduction)	ROS formation, GST activity, and reproduction		Lim et al. 2012
Ag	50.3 nm (citrate-coated)	*Caenorhabditis elegans*	10 - 1000 mg/L (survival) and1 – 10 mg/L (reproduction)	Agar and K-media	24h (survival) and 48h (reproduction)	Survival and reproduction	LC50 =55mg/L and EC50=>100mg/L in agar	Kim et al. 2012

Nanoparticles: Ag= silver; Al_2O_3= aluminium oxide; Au= gold; CeO_2= cerium oxide; Cu= copper; TiO_2= titanium oxide; ZnO= zinc oxide.
Endpoints: ROS = reactive oxygen species; GST = glutathione S-transferases enzyme

Table 2-1: Continued

NPs	Initial size	Species tested	Concentration ranges	Exposure media	Duration	Endpoints	Outcomes	Reference
Al_2O_3	60 nm	*Caenorhabditis elegans*	10.2 - 407.8 mg/L	Water	24h	Survival and feeding inhibition	LC50= 82 mg/L	Wang et al. 2009
Al_2O_3	11 nm	*Eisenia fetida*	100- 10,000 mg/kg	Field soil Grenada-Loring	28 d, and 2 d (avoidance)	Survival, reproduction and avoidance		Coleman et al. 2010
Au	20 and 50 nm	*Eisenia fetida*	5-50 mg Au/kg dry mass	Artificial soil	28 d	Survival, growth reproduction and gene expression		Unrine et al. 2010a
CeO_2	15 and 45 nm	*Caenorhabditis elegans*	1 mg/L	K-media	24h	Survival, growth reproduction and gene expression		Roh et al. 2010
Cu	20-40 nm and <100 nm	*Eisenia fetida*	5- 50 mg Cu/kg d.w.	Artificial soil	28 d, and 56 d (reproduction)	Survival, growth, reproduction and gene expression		Unrine et al. 2010b
Cu	80 nm	*Enchytraeus albidus*	400 - 1000 mg Cu/kg	Field soil	48h	Gene expression		Gomes et al. 2012

Nanoparticles: Ag= silver; Al_2O_3= aluminium oxide; Au= gold; CeO_2= cerium oxide; Cu= copper; TiO_2= titanium oxide; ZnO= zinc oxide.
Endpoints: ROS = reactive oxygen species; GST = glutathione S-transferases enzyme

Table 2-1: Continued

NPs	Initial size	Species tested	Concentration ranges	Exposure media	Duration	Endpoints	Outcomes	Reference
Cu	80 nm	*Enchytraeus albidus*	130 and 230 mg Cu/kg	Field soil	3 and 6 weeks	Energetic reserves	EC10 (Lipid reduction) = 97 mg Cu NP/kg, EC10 (protein reduction) = 157 mg Cu NP/kg	Amorim et al. 2012
TiO_2	50 nm	*Caenorhabditis elegans*	24.0 - 239.6 mg/L	Water	24h	Survival, growth, reproduction and feeding	LC50= 80 mg/L	Wang et al. 2009
TiO_2	7 and 20 nm	*Caenorhabditis elegans*	1 mg/L	K-media	24h	Survival, growth reproduction and gene expression		Roh et al. 2010
TiO_2	15 nm	*Porcellio scaber*	0.1-3,000 µg TiO2/g dry leaf	Hazelnut tree leaves	3 d	Survival and feeding inhibition		Jemec et al. 2008
TiO_2	<25 nm and <75 nm	*Porcellio scaber*	10-1,000 mg2/g dry food	Hazelnut tree leaves	14 d	Survival and feeding inhibition		Drobne et al. 2009

Nanoparticles: Ag= silver; Al_2O_3= aluminium oxide; Au= gold; CeO_2= cerium oxide; Cu= copper; TiO_2= titanium oxide; ZnO= zinc oxide.
Endpoints: ROS = reactive oxygen species; GST = glutathione S-transferases enzyme

Table 2-1: Continued

NPs	Initial size	Species tested	Concentration ranges	Exposure media	Duration	Endpoints	Outcomes	Reference
TiO_2	10×50 nm (Al(OH) 3 and polydim ethyl siloxane coated)	*Lumbricus terrestris*	1-100 mg/L (water), 10 and 100 mg/kd (dry food) and 15 mg /kg (dry soil)	Water, ground horse manure and natural soil	7 d	Mortality and frequency of apoptosis		Lapied et al. 2011
TiO_2	5, 10 and 21 nm	*Eisenia fetida and E. Andrei*	20-10,000 mg/kg	Field and artificial soil	14 d (survival), 18 weeks (growth), 28 d (reproduction) and 48 h(avoidance)	Survival, growth, reproduction and avoidance		McShane et al. 2012
TiO_2	32 nm	*Eisenia fetida and E. Andrei*	0.1 – 10,000 mg/L (survival) and 0.1 – 1,000 mg/kg (reproduction)	Filter paper, sand and artificial soil	14 d	Survival and reproducyion		Cañas et al. 2011
ZnO	<50 nm	*Folsomia candia*	230 mg Zn/kg dry soil	OECD artificial soil	28 d	Survival and reproduction		Manzo et al. 2010

Nanoparticles: Ag= silver; Al_2O_3= aluminium oxide; Au= gold; CeO_2= cerium oxide; Cu= copper; TiO_2= titanium oxide; ZnO= zinc oxide.
Endpoints: ROS = reactive oxygen species; GST = glutathione S-transferases enzyme

Table 2-1: Continued

NPs	Initial size	Species tested	Concentration ranges	Exposure media	Duration	Endpoints	Outcomes	Reference
ZnO	<100 nm	*Folsomia candida*	100 – 6400 mg Zn/kg dry soil	LUFA soil	28 ds	Survival and reproduction	LC50 = >3086 mg/kg; EC50 = 1964 mg/kg	Kool et al. 2011
ZnO	<100 nm	*Eisenia veneta*	6 to 96 mg Zn/L DI water	DI water (immersion test)	24 h	Survival	LC50 = 1.75 mg/L (CI = 1.21 - 3.44 mg/L)	Hooper et al. 2011
ZnO	<100 nm	*Eisenia veneta*	250 and 750 mg/kg dry soil	Clay loam soil	21 d	Survival, immune activity, and life history trait	750 mg/kg ~ Reproduction EC30,	Hooper et al. 2011
ZnO	1-2.5 nm	*Caenorhabditis elegans*	10–1,625 mg Zn/L	Water	24h, and 72h (reproduction)	Survival, reproduction, mobility, genetic	LC50= 800 mg/L, EC50(reproduction) = 46 mg/L, EC50(mobility) = 600 mg/L	Ma et al. 2009
ZnO	<100 nm	*Porcellio scaber*	2000 and 5000 µg/g dry food	Hazelnut tree leaves	28 d	Survival and feeding inhibition		Pipan-Tkalec et al. 2010

Nanoparticles: Ag= silver; Al_2O_3= aluminium oxide; Au= gold; CeO_2= cerium oxide; Cu= copper; TiO_2= titanium oxide; ZnO= zinc oxide.
Endpoints: ROS = reactive oxygen species; GST = glutathione S-transferases enzyme

Table 2-1: Continued

NPs	Initial size	Species tested	Concentration ranges	Exposure media	Duration	Endpoints	Outcomes	Reference
ZnO	20 nm	*Caenorhabditis elegans*	0.4 - 8.1 mg/L znO-NPs	Water	24h	Survival, growth, reproduction and feeding	LC50= 2.2 mg/L	Wang et al. 2009
ZnO	40-100 nm	*Eisenia fetida and E. Andrei*	0.1 – 10,000 mg/L (acute) and 0.1 – 1,000 mg/kg (reproduction)	Filter paper, sand and artificial soil	14 d	Survival and reproduction		Cañas et al. 2011

Nanoparticles: Ag= silver; Al_2O_3= aluminium oxide; Au= gold; CeO_2= cerium oxide; Cu= copper; TiO_2= titanium oxide; ZnO= zinc oxide.
Endpoints: ROS = reactive oxygen species; GST = glutathione S-transferases enzyme

Conclusion

Effects of NPs on invertebrate organisms often yield conflicting results, when regarding different LC50 values obtained (Darlington et al., 2009; Wang et al., 2009), or whether toxicity is caused by NPs or dissolved ions (Kool et al., 2011; Manzo et al., 2010). Often such conflicts may simply be due to differences in the properties of the NPs and test media employed due to their complex interaction and effect on the NP fate and behavior. Consequently, many times no clear conclusions can be drawn with the level of exposure characterisation provided or possible in soil, and NPs toxicity remains a point of concern requiring further evaluation.

Nevertheless, some remarks can be made about NPs toxicity in soils: (a) the presence of NPs in the organisms' tissue indicates the assimilation of nanosized particles is possible (Hooper et al., 2011; Unrine et al., 2010a); (b) nanoparticle core elements clearly influence toxicity, for example TiO_2 was found to be less toxic than Ag (McShane et al., 2011) and ZnO NPs (Cañas et al., 2011); (c) tests should be conducted in media which are as close to realistic conditions as possible to have relevance and enable a better understanding of NPs toxicity in the environment (e.g., K-media leads to greater aggregation and is more similar to the pore water conditions than agar (Kim et al., 2012)); (d) care should be taken when interpreting non-soil media (e.g. liquids or agar) for testing as novel modes of action, like photo-induced ROS generation for e.g. TiO_2, ZnO, and CeO_2 (Cañas et al., 2011; Roh et al., 2010), can lead to effects that are not majorly relevant in soil organisms, no matter how interesting and novel they are; (e) toxicity can be due to metal ions and NPs or just one of the components and therefore dissolution studies can help deriving conclusions regarding the actual cause of toxic effects seen.

Moreover, advances in characterization methodologies of NPs in solid media will provide a crucial contribution to the development of (eco)toxicological knowledge for NPs. Being able to relate the outcomes of routine available physical and chemical analysis that better describes NP exposure in fundamental detail within soils (e.g. Agglomeration state and dissolution rates) to ecotoxicological test

results are urgently needed to support answering questions on the role of NPs in the toxicity to soil organisms, which is not possible at the present time.

2.7 Overview and conclusion

The term metal-based NPs encompasses many different types of particles (e.g., various core metals as either pure metals, oxide, or other chemical coordination) resulting in great variations regarding their environmental behavior, bioavailability and toxic effects. Even NPs with the same core material may exhibit a range of behaviors depending on factors such as coating material, shape, and size. Also, different environmental release patterns will result from the manner in which the NPs are incorporated in the final products, as nanoparticles can be either fixed in a substrate, such as polymers, composites, resins, coatings, or included in formulations (e.g., creams and liquid products), or in rare cases as free particles (Bystrzejewska-Piotrowska et al., 2009). Physicochemical conditions during wastewater treatment processes and in the receiving environmental medium (e.g., water or soil) are as important as the original particle properties. They are responsible for modifications of the particles (e.g., surface charge and zeta potential changes caused by different pH levels), affecting processes governing NP fate and behavior (e.g., aggregation/agglomeration, dissolution). Therefore, environmental parameters should be measured in ecotoxicological tests, as they are likely to be important controls on NP bioavailability. It is a challenge to interpret information obtained from toxicological tests, as small changes in so many factors can lead to different results. The study by El-Badawy et al. (2011), for instance, clearly demonstrated an important influence of surface charge on toxicity.

Ecotoxicological tests should attempt to reproduce the real behavior of NPs in the soil in order to robustly assess the potential effects on soil organisms. For this purpose, test designs and exposure characterisation measurements need to be formulated with the factors likely to influence NP bioavailability in mind. The tests should of course be conducted covering a wide range of concentrations to fully comprehend toxicity in different scenarios and to provide LC50s and EC50s values for hazard assessment. Also, it is important to determine what metric provides the best measure of dose and whether a response is dose-dependent. However, it

must be borne in mind that NPs will show dose-dependent behavior in soils as they do in other media, e.g., processes like high dose agglomeration may lead to drops in overall surface area and slowing down of dissolution.

The characterization of NPs in soil media is a challenging field currently involving expensive techniques. As a consequence, the majority of published ecotoxicological studies examine and report NP characteristics only in stock solutions used. However, it must be remembered that this provides very little information on NP characteristics in the soil. Electron microscopy techniques and chemical analyses are essential for characterizing the NPs (i.e., aggregation, particle size, and shape) and the levels of free ions released (i.e., dissolution). Nanoparticle characterization should be conducted as much as possible in the exposure medium and not only in the stock solutions used to spike the soil. It is realized, however, that extractions may greatly affect the parameters of interest. Only very advanced and expensive techniques like bulk EXAFS and XANES can really achieve proper in situ characterization of NPs in soil. Therefore, often more routine characterization techniques like analysis of extracted soil pore water must be relied upon. While DLS techniques are becoming a standard for this, much more information can be gained from single particle ICP-MS analysis (including any soil components bound to the NPs). If coupled with field-flow fractionation (FFF) and other separation techniques, the associated hydrodynamic size and charge of particles can be assessed. In addition, parameters known to affect the behavior of NPs, such as pH, ionic strength, and soil organic matter content, should be measured to facilitate comparison across studies and maximise the future potential use of the data generated.

The stability of NPs is also time-dependent and thus NP toxicity will depend on test duration. Long-term effects will depend on the fate of NPs, driven by processes such as dissolution and the interactions of both NP surfaces and metal ions (generated by dissolution) with the matrix components (e.g., humic acids). Efforts focusing on linking the development of NP fate in time (aging) and toxicity at realistic low doses should be encouraged.

Studies of metal-based NP effects should further take into account the existing knowledge on the effects of metals on soil organisms (van Gestel et al., 2010).

The authors highlighted that some metals are essential (e.g., zinc), and thus test organisms should contain a minimum metal concentration in the body to be considered “healthy” that may be regulated to a constant level even following increased exposure and uptake. At the same time, the metal concentration should not exceed toxic levels (van Gestel et al., 2010). The exposure time is important not only for NP dissolution, but also for NP toxicokinetics (i.e., NPs assimilation and elimination) (van Gestel et al., 2010).

Comparing the outcomes from the literature is difficult given the inconsistencies among studies (Table 2-1). The great variability of NP characteristics (e.g., size, shape, and coating), the way they are produced and functionalized and the differences in test designs (e.g., exposure media, spiking procedure, and duration) and endpoints make it challenging to draw general conclusions about nanoparticle toxicity to soil organisms. Answering this challenge calls for more standardization of tests on bioaccumulation and toxicity of NPs to soil invertebrates and other organisms.

As a first step, as much as possible must be learned from studies on the behavior of natural nanoparticles in natural samples to provide baseline data for interpretation, before manufactured NPs reach levels in the environment where they can be monitored routinely. It should be remembered there is a rich literature within the natural colloid sciences here as well as on going new research. Moreover, transformations of nanoparticles prior to and following entry to the environment, such as surface coating with humic acids, interactions with common cations and complexes, and dissolution under natural conditions, may be important in controlling behavior and fate and require further research. Parallel physicochemical/toxicity studies are needed to gain a more systematic understanding of how properties influence nanoparticle bioavailability and toxicity.

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CHAPTER 3: INFLUENCE OF SOIL PH ON THE TOXICITY OF ZINC OXIDE NANOPARTICLES TO THE TERRESTRIAL ISOPOD *Porcellionides pruinosus*

Paula S. Tourinho[1], Cornelis A.M. van Gestel[2], Stephen Lofts[3], Amadeu M.V.M. Soares[1], and Susana Loureiro[1]

[1]Department of Biology & CESAM, University of Aveiro, 3810 193 Aveiro, Portugal

[2]Department of Ecological Science, Faculty of Earth and Life Sciences, VU University, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

[3]CEH Lancaster, Lancaster Environment Centre, Lancaster, LA1 4AP, United Kingdom

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3.1 Abstract

The effects of soil pH on the toxicity of ZnO nanoparticles (NPs) to the terrestrial isopod *Porcellionides pruinosus* were evaluated. Isopods were exposed to a natural soil amended with $CaCO_3$ to reach three different pH_{CaCl2} levels (4.5, 6.2 and 7.3) and standard Lufa 2.2 soil (pH 5.5) spiked with ZnO NPs (30 nm), non-nano ZnO (200 nm) and ionic Zn as $ZnCl_2$. Toxicity was expressed based on total Zn concentration in soil, and total Zn and free Zn^{2+} ion concentrations in pore water. Compared to ZnO spiked soils, the $ZnCl_2$ spiked soils had lower pH and higher porewater Ca^{2+} and Zn levels. Isopod survival did not differ between Zn forms and soils, but survival was higher for isopods exposed to ZnO NPs at pH 4.5. Median effect concentrations (EC50) for biomass change showed similar trend for all Zn forms in all soils, with higher values at intermediate pH. LC/EC50 values based on porewater Zn or free Zn ion concentrations were much lower for ZnO than for ionic zinc. Zn body concentrations dose-related increased, but no effect of soil pH was found. It is suggested that not only dissolved or free Zn in pore water contributed to uptake and toxicity, but that oral uptake (i.e. ingestion of soil particles) could be an important additional route of exposure.

Keywords: ZnO nanoparticles, soil pH, isopods, toxicology, bioavailability

3.2 Introduction

Manufactured or engineering nanoparticles (NPs) have attracted industrial and scientific interests in the last decade because of their unique properties. Innovative products, used in diverse fields, have resulted in a substantial investment in the nanotechnology sector, which is estimated to be $1 trillion in 2015 (Roco, 2011). Because of the increasing annual production over the years, NPs have been regulated by REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) in Europe, under the same legislation as bulk compounds, even though nano and bulk materials present different properties (Bondarenko et al., 2013).

Zinc oxide (ZnO) NPs are among the most produced NPs, with production volumes of more than 500 tonnes/year in 2010 (Piccinno et al., 2012). ZnO NPs are mainly used in cosmetics (as UV absorbants in sunscreens), paints and coatings (Piccinno et al., 2012). Use of NPs may result in emission to the environment, with soil being an important sink (Chapter 2).

Some attention has been given to the behavior and effects of nanoparticles in the environment (see Chapter 2). The processes of dissolution and aggregation/agglomeration have shown to be dependent on characteristics of both the media of exposure and the NPs. The stability of ZnO NPs is affected by environmental conditions such as pH (Ben-Moshe et al., 2010), organic matter content (Jiang et al., 2012a) and ionic strength (Jiang et al., 2012b). In soils, the pH is one of the most important factors to consider in toxicity tests, because it can change the NP surface charge and zeta potential (Chapter 2). As a consequence the interactions between NPs and the soil as well as the interactions between particles will change, influencing NP behavior, bioavailability and toxicity.

The effect of soil pH on the bioavailability of ionic zinc to soil organisms has already been studied. Zinc toxicity to the potworm *Enchytraeus albidus* decreased with increasing soil pH (Lock and Janssen, 2003). For the springtail *Folsomia candida*, EC50 for effects on reproduction was lower in an acid soil than in a basic soil (pH_{KCl} of 3.4 and 6.0, respectively), and toxicity was mainly related to the water-extractable Zn fraction (Smit and Van Gestel, 1996). In another study (Sandifer and Hopkin, 1996), the reproduction of *F. candida* decreased with

decreasing pH (ranging from 4.5 to 6.0), but no clear relation between toxicity and soil pH was found. For the earthworm *Lumbricus rubellus*, soil pH did not affect zinc accumulation in the body, however, reproduction was affected by soil pH, being related to the soluble Zn fraction (Spurgeon et al., 2006). In this study, toxic responses could be predicted by free Zn^{2+} concentration and explained by the protective effect of H^+ ions (i.e. competition with Zn^{2+} ions) (Spurgeon et al., 2006), which seems to agree with the Biotic Ligand Model (Thakali et al., 2006).

The bioavailability and toxicity of ZnO NPs has been evaluated for collembolans (Kool et al., 2011; Manzo et al., 2011; Waalewijn-Kool et al., 2012) and earthworms (Hooper et al., 2011) by comparing the outcome with micro-size ZnO and/or ionic Zn forms. For terrestrial isopods, the toxicity of ZnO NPs has been assessed using contaminated food as the route of exposure (Pipan-Tkalec et al., 2010). Soil is also an important route of exposure to chemicals for isopods, and should also be investigated for NPs (Fischer et al., 1997; Loureiro et al., 2009; Sousa et al., 2000). Isopods can either take up chemicals from soil by ingesting soil particles or by pore water inflow through the uropods. The influence of environmental conditions, such as pH, on the bioavailability of NPs in soils is an important issue and is far from being completely understood (Chapter 2; van Gestel, 2012).

This study therefore aimed at evaluating the effects of soil pH on the toxicity of ZnO NPs to the terrestrial isopod *Porcellionides pruinosus*. For this purpose, a natural soil from Dorset (United Kingdom) was amended with $CaCO_3$ to reach three different pH levels. A standard soil (Lufa 2.2) was included for comparison. In order to better understand the contribution of particle size and ionic zinc to the toxicity of ZnO-NP to the isopods, toxicity tests were also conducted with a micro-size ZnO and $ZnCl_2$.

3.3 Methodology

Soil treatment

Natural soil was collected at Wareham forest (Dorset, United Kingdom) in May 2011. Soil was excavated from the 0-30 cm top soil layer. The soil originally had a pH_{CaCl2} of 3.0. After sieving (5 mm mesh) and air-drying, the soil was amended

with calcium carbonate to adjust pH_{CaCl2} to nominal values of 4.5 (Soil 1), 5.9 (Soil 2), and 7.3 (Soil 3). The standard Lufa 2.2 soil (LUFA-Speyer 2.2, Sp 2121, Germany, 2009) was also used in the experiment. For details on pH adjustment see Heggelund et al. (2014). Maximum Water Holding Capacity (WHCmax) of the Dorset soils was approx. 77%, and of LUFA 2.2 soil it was 45%. Table 3.1 presents the soil properties and pH levels of the different test soils.

Table 3-1: Properties of Dorset soil with different amounts of calcium carbonate (w/w%) added and standard Lufa 2.2 soil. Data refers to nominal and actual measured pH values, organic matter content and cation exchange capacity (CEC) (mean ± standard deviation; n=2) before spiking the soils.

	$CaCO_3$ (w/w%)	Nominal pH_{CaCl2}	Measured pH_{CaCl2}	Organic matter (%)	CEC ($cmol_c$/kg soil)
Soil 1	0.20	4.5	4.5	7.39 ± 0.00	8.19 ± 0.74
Soil 2	0.45	5.9	6.2	7.63 ± 0.14	9.09 ± 0.05
Soil 3	1.00	7.2	7.3	7.65 ± 0.27	10.8 ± 0.73
Lufa 2.2 soil	-	-	5.1	4.35± 0.09	8.24 ± 0.34

The soils were spiked with zinc oxide NPs (Nanosun ZnO P99/30; particle size 30 nm), non-nano ZnO (Microsun ZnO W45/30; 200 nm), and zinc chloride ($ZnCl_2$; Riedel-de Haën; purity 98%) at nominal concentrations of 250, 500, 1,000, 2,000 and 4,000 mg Zn/kg dry soil. To spike the soils with ZnO, the dry powders were added to 30 g of dry soil. After thorough mixing, the mixture was added to 270 g of dry soil. Milli-Q water was added to achieve a moisture content corresponding with 45% of the WHC_{max} of the soils. For $ZnCl_2$, 300 g of dry soil was mixed with a $ZnCl_2$ solution in Milli-Q water. If necessary, additional Milli-Q water was added to moisten the soil up to 45% of WHC_{max}. Non-spiked soils were moistened with Milli-Q water and tested as control soils. Soils were allowed to equilibrate for one week before starting the toxicity tests.

Toxicity test

Specimens of the isopod *Porcellionides pruinosus* were collected in Coimbra (Portugal) and kept under laboratory conditions for at least one month before exposure. The animals were kept on a substrate of potting soil with alder (*Alnus glutinosa*) leaves provided *ad libitum* for food. Males and non-gravid females (>12 mg) were exposed individually in plastic boxes containing 20 g of moist soil. Ten replicates were used for each treatment and control. Dry alder leaf disks (Ø 10 mm) were offered to the isopods as food *ad libitum*. The animals were kept at 20±1ºC and a light:dark photoperiod of 16:8 h. Water loss was checked and adjusted after 7 days by weighing the test containers. After 14 days, survival and feeding activity were evaluated. The parameters used in this experiment were the consumption ratio and biomass change calculated as:

$$Cr = (W_{li} - W_{lf}) / W_{isop} \quad (1)$$

$$B = (W_{isopf} - Wi_{sop}) / W_{isop} * 100 \quad (2)$$

where, Cr is the consumption ratio (mg leaf/mg isopod), W_{li} the initial leaf weight (mg dw), W_{lf} the final leaf weight (mg dw), W_{isop} the initial isopod weight (mg fw), B the biomass change (%), and W_{isopf} the final isopod weight (mg fw).

Chemical analysis

Soil pH was measured in 0.01 M $CaCl_2$ extracts at the beginning of the test, in accordance to ISO guideline 10390 (ISO, 1994). For determining total Zn concentrations in soil, dry soil samples were digested for 7 hours in a mixture of ultrapure water, concentrated HCl (J.T. Baker, purity 37%) and HNO_3 (J.T. Baker, purity 70%) (1:1:4, v/v) at 140ºC in an oven (CEM MDS 81-D). After digestion, the samples were analysed for zinc by flame atomic absorption spectrometry (AAS) (Perkin-Elmer AAnalyst 100). Certified reference material (ISE sample 989 of River Clay from Wageningen, The Netherlands) was used to ensure the accuracy

of the analytical procedure. Measured zinc concentrations in the reference material were within 10% of the certified concentrations.

Pore water was collected by saturating 50 g of soil with ultrapure water for one week. Samples were centrifuged at 4,000 rpm for 90 min (Eppendorf 5810R). The supernatant was collected and filtered using a cellulose nitrate filter (Whatman, 0.45 µm pore size). Total zinc concentration in pore water was analyzed by flame AAS (Perkin-Elmer AAnalyst 100), after dilution with distilled water. Calcium concentrations in the porewater samples were determined after dilution with 1% $La(NO_3)_3$ in 0.1 nHNO_3 and analyzed by flame AAS (Perkin-Elmer AAnalyst 100).

After 14 days of exposure, total zinc body content in the surviving isopods was analyzed in triplicates for each exposure concentration. After freeze-drying, isopods were individually weighted and digested with a mixture of concentrated HNO_3:$HClO_4$ (7:1, v/v, J.T. Baker, ultrapure). The samples were evaporated to dryness and the residues were taken up in 300 µl 0.1 M HNO_3. Zinc content was determined by graphite furnace AAS (Perkin-Elmer 5100 PC).

Statistical analysis

Zinc concentrations causing 50% mortality (median lethal concentrations or LC50s) of *P. pruinosus* were calculated by probit analysis. Consumption ratio (log-transformed) was analyzed by one-way analysis of variance (ANOVA), followed by a Dunnett's test. Data homoscedasticity and normality were tested by Levene's test and Kolmogorov-Smirnov test, respectively. For biomass change, median effect concentrations (EC50s) were estimated by applying a four parameter logistic model:

$$Y = Ymin+(Ymax-Ymin)/(1+(X/EC50)^{-b}) \quad (3)$$

where Ymin is the minimum biomass gain (%); Ymax the maximum biomass gain (%); X the Zn concentration in soil (mg Zn/kg) or pore water (mg Zn/L), or the free Zn^{2+} ion concentration (µM) in pore water; and b the slope parameter. The free Zn ion concentrations were estimated from total Zn concentrations in pore water using the speciation model WHAM7.

Slopes of the probit regression and EC50 values were compared between the different soils by a generalized likelihood ratio test. Zinc body content in the isopods was analyzed by a two-way ANOVA, using zinc concentration in soil and soil pH as the independent variables. When necessary, data was log-transformed to reach homoscedasticity and normality. Statistical analyses were performed with SPSS software (version 20).

3.4 Results

3.4.1 Soil characteristics

Soil pH changed in the presence of zinc in all soils, mainly in a dose-related manner. A great difference was found between soils spiked with ZnO particles (30 nm and 200 nm ZnO) and $ZnCl_2$ (Figure 3.1). For ZnO particles, the pH increased up to 2 units with increasing Zn concentration in soils 1, 2, and Lufa soil, while a slight dose-related decrease of up to 0.3 pH units at the highest test concentration was found in soil 3. For $ZnCl_2$, in all soils pH decreased in a dose-related fashion up to 0.9 units at the highest Zn concentration. Porewater pH levels showed the same trends as soil pH (see Supplementary Material, Table S 3-1).

Total measured Zn concentrations in the soil ranged between 68 and 130% of the nominal ones (see Table S 3-1). All effect concentrations reported are based on measured concentrations.

Calcium concentrations in pore water also varied between the different Zn forms and concentrations (Figure 3.1). For 30 nm and 200 nm ZnO, calcium levels remained approximately constant in soils 1 and 2, ranging between 11.7 and 22.1 mg Ca/L. In soil 3, calcium levels slightly decreased with increasing ZnO concentration in soil, while in Lufa 2.2 soil, they slightly increased. For $ZnCl_2$, calcium levels increased with increasing Zn concentration and were between 30 and 50 times higher than in Dorset soils and 10-fold higher than in Lufa soil spiked with ZnO.

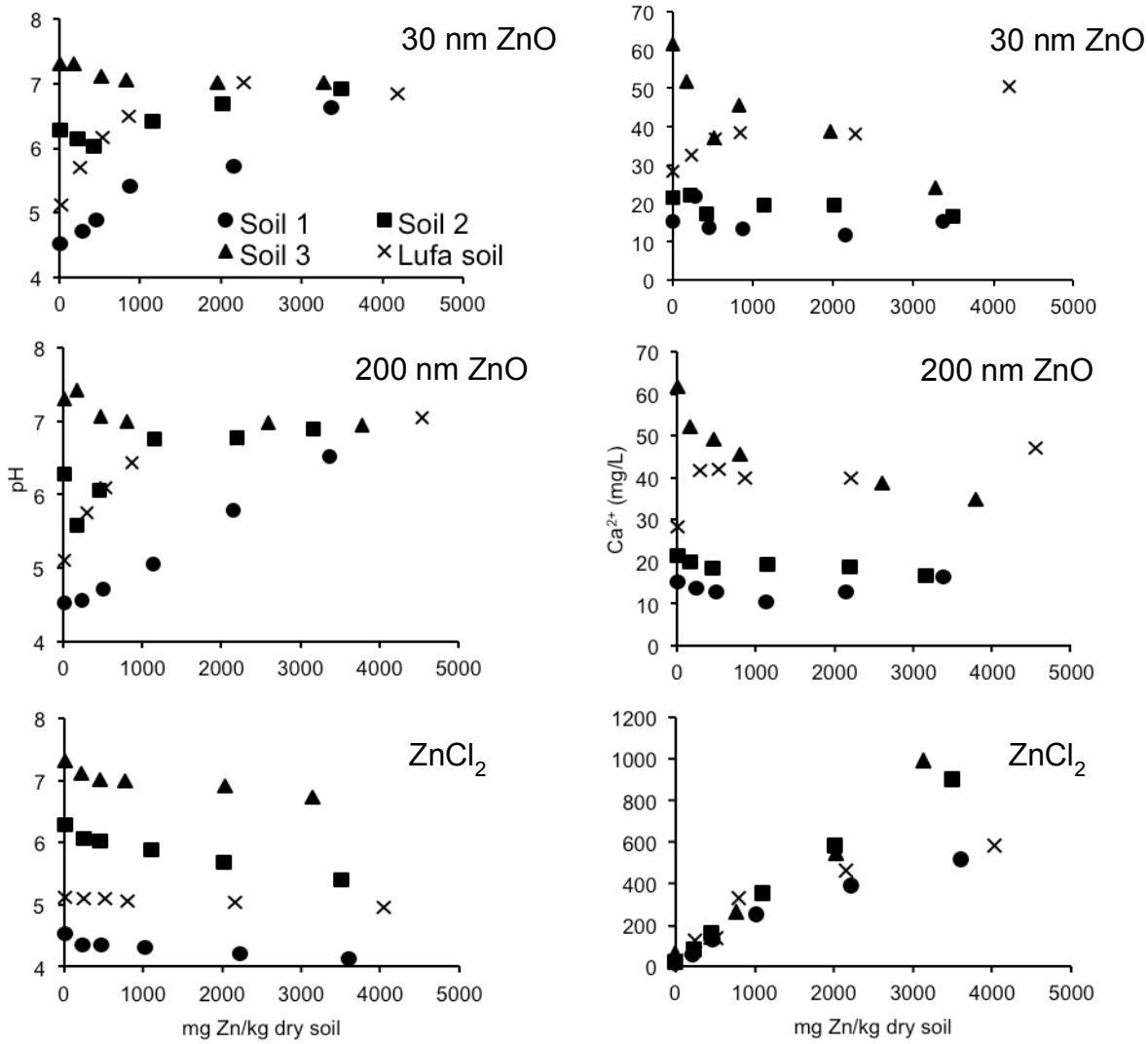

Figure 3.1: Soil pH_{CaCl2} and calcium (Ca^{2+}) levels (mg/L) in pore water of soils spiked with 30 nm ZnO, 200 nm ZnO and $ZnCl_2$. See Table 1 for soil properties.

Zinc concentrations in pore water were similar in soils spiked with nano- and micro-sized ZnO particles, showing a slight increase with increasing Zn concentration (Table S 3-1). $ZnCl_2$-spiked soils showed strong dosed-related increase of Zn levels in pore water, reaching concentrations ~100-fold higher than in ZnO-spiked soils.

3.4.2 Toxicity to *Porcellionides pruinosus*

Mortality

Control survival of isopods was 80, 100, 90, and 100% for soil 1, 2, 3 and Lufa 2.2 soil, respectively. LC50 values could be calculated for all three Zn forms in the

different soils, except for 30 nm ZnO in soil 1 where only 30% mortality occurred at the highest concentration. LC50 values for the effects of 30 nm ZnO on survival of the isopods ranged from 1,757 to >3,369 mg Zn/kg dry soil in the different soils (Table 3.2). LC50 values ranged from 2,169 to 2,894 mg Zn/kg dry soil and from 1,792 to 3,732 mg Zn/kg dry soil for 200 nm ZnO and $ZnCl_2$, respectively (Table 3.2). LC50 values decreased with increasing soil pH for ZnO NPs (Table 3.2). No significant difference in slopes of the probit regressions were found between soils for 30 nm ZnO ($X^2_{(2)}$= 5.56, *p*>0.05), 200 nm ZnO ($X^2_{(3)}$=0.86, p>0.05), and $ZnCl_2$ ($X^2_{(3)}$=7.68, p>0.05).

LC50 values were also calculated based on Zn concentration (mg/L) and free Zn^{2+} ion concentration in pore water (µM) for $CaCO_3$-amended Dorset soils (Supplementary Material, Tables S 3-2 and S 3-3). Values found for ZnO were found to be much lower than for $ZnCl_2$, ranging from 1 to 32 µM and 2,000 to 24,000 µM for ZnO and $ZnCl_2$, respectively.

Dead animals were excluded from further analysis of sublethal responses and Zn body content.

Feeding inhibition

Feeding activity measured as consumption ratio of control animals differed significantly between soils (ANOVA, p<0.05). In soil 3, consumption ratio was significant higher than in all other soils, while in soil 1 consumption ratio was significantly higher than in Lufa 2.2 soil (ANOVA, p<0.05). Consumption ratio did not change in the isopods exposed to 30 nm and 200 nm ZnO in soils 1, 2 and Lufa 2.2 soil (ANOVA, p>0.05). However, consumption ratio decreased significantly in soil 3 at 2,000 and 1,000 mg Zn/kg for 30 nm and 200 nm ZnO, respectively (Dunnett's test, p<0.05; Figure 3.2). Due to high mortality at these concentrations, the sample sizes were 3 and 1 at 2,000 and 4,000 mg Zn/kg, respectively for 30 nm ZnO, and 8, 2 and 2 at 1,000, 2,000 and 4,000 mg Zn/kg, respectively for 200 nm ZnO. For $ZnCl_2$, consumption ratio was dose-related decreased in all tested soils at concentrations ≥ 500 mg Zn/kg (Dunnett's test, p<0.05).

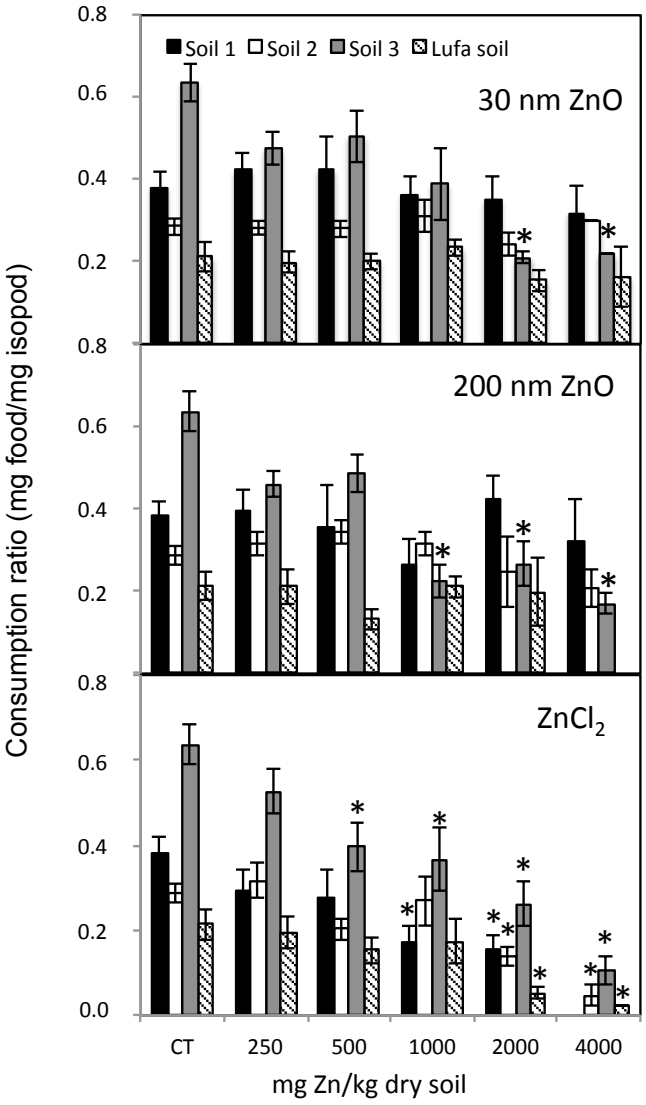

Figure 3.2: Food consumption (expressed as consumption ratio in mg food/mg isopod) of the isopod *Porcellionides pruinosus* exposed to different concentrations of 30 nm ZnO NPs, 200 nm ZnO, and $ZnCl_2$ in four different soils after two weeks (CT = control soil; Soil 1 – pH 4.5; Soil 2 – pH 6.2; Soil 3 – pH 7.3; Lufa 2.2 soil – pH 5.1). * Represents significant differences with Dunnett's test ($p<0.05$).

Biomass change

Biomass change of the isopods, calculated as the difference between final and initial fresh weights, did not differ between control soils (ANOVA, $p>0.05$). EC50s ranged from 713 to 1,479 mg Zn/kg for 30 nm ZnO, from 119 to 1,951 mg Zn/kg for 200 nm ZnO and from 331 to 1,478 mg Zn/kg for $ZnCl_2$ (Table 3-2). The corresponding logistic dose-response relationships based on total Zn

concentrations can be found in Figure S 3-1. The three Zn forms showed similar trends in EC50 values with soil pH. Soil 2 showed the highest EC50 values for all Zn forms, however, significant differences between EC50 were only found for 200 nm ZnO ($X^2_{(3)}$=69.82, p<0.001) and $ZnCl_2$ ($X^2_{(3)}$=23.10, p<0.001).

Table 3-2: LC50 and EC50 (mg Zn/kg dry soil) values for the effects of 30 nm ZnO NPs, 200 nm ZnO and $ZnCl_2$ on the survival and biomass change of *Porcellionides pruinosus* in four different soils. All values are based on measured Zn concentrations; 95% confidence intervals are given in between brackets. See Table 1 for soil properties.

	LC50			EC50		
	30 nm	200 nm	$ZnCl_2$	30 nm	200 nm ZnO	$ZnCl_2$
Soil 1	>3,369	2,277 (1,505-4,334)	2,352*	713[a] (127-1,300)	119 [a] *	312[a] (97-528)
Soil 2	2,586*	2,551 (2,017-3,491)	3,732 (3,013-6,751)	1,479[a] (913-2,046)	1,951[b]*	1,400[b] (886-1,913)
Soil 3	1,757 (1,339-2,351)	2,169 (1,628-2,899)	1,792*	904[a] (533-1,274)	974[c]*	783[c]*
Lufa 2.2	3,361 (2,593-4,839)	2,894*	2,292 (1,698-3,229)	788[a] (117-1,458)	1,405[bc] (670-2,141)	687[abc] (332-1,042)

* not possible to calculate reliable 95% confidence intervals.
[a,b,c] Significant differences between EC50 values for the different soils as determined by a generalized likelihood ratio test.

EC50 values for effects on biomass change based on total porewater Zn concentrations ranged from 4.21 to 9.06 mg Zn/L and 2.28 to 3.23 mg Zn/L for 30 and 200 nm ZnO, respectively. For $ZnCl_2$, EC50 values ranged from 35.9 to 250 mg Zn/L (Table S 3-2). Biomass change in isopods exposed to ZnO particles was not dose-related to free Zn^{2+} ion concentration in the pore water of soils 1 and 2, making it therefore impossible to obtain EC50s based on Zn^{2+} concentration. In soil 3, EC50 values were 0.59 µM and 0.42 µM for 30 nm and 200 nm ZnO, respectively. For $ZnCl_2$, EC50 values were 449, 3,000, and 37.8 µM in soils 1, 2 and 3, respectively (Table S 3-3). Logistic dose-response relationships based on

total soil Zn concentrations, and on total Zn and free Zn^{2+} concentrations in the pore water can be found in Figure S 3-2.

Zinc body content

Zinc body content in the isopods showed a dose-related increase in all soils and for all three Zn forms tested (Figure 3.3). Zn body concentrations of isopods exposed to 30 nm ZnO were affected by zinc concentration in soil (ANOVA, $p<0.01$), but not by soil pH (ANOVA, $p>0.05$) or the interaction between soil concentration and pH (ANOVA, $p>0.05$). Similarly, for 200 nm ZnO, a significant effect of soil concentration on Zn body content of the isopods was observed (ANOVA, $p<0.01$) with no significant effect of soil pH or their interaction (ANOVA, $p>0.05$). For $ZnCl_2$, zinc body content significantly increased with soil concentration (ANOVA, $p<0.01$) and soil pH (ANOVA, $p<0.05$), however the interaction was not significant (ANOVA, $p>0.05$). In $ZnCl_2$ exposed isopods, zinc body content differed between soil 1 and Lufa soil (Tukey test, $p<0.05$).

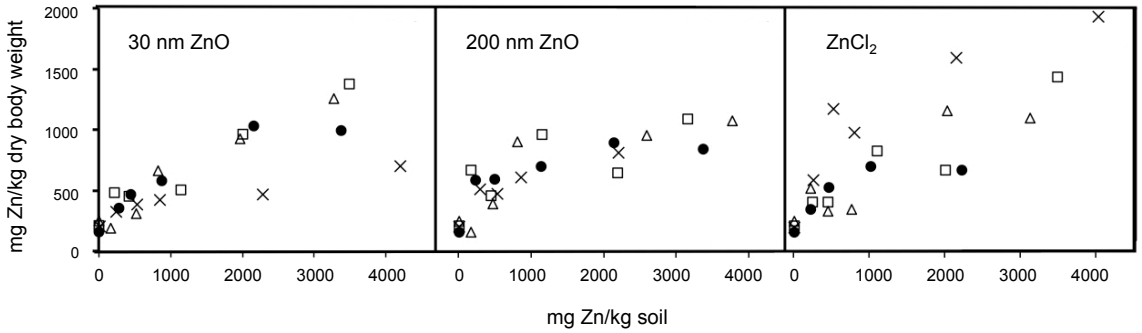

Figure 3.3: Zinc body concentrations (mg Zn/kg dry body weight) of the isopod *Porcellionides pruinosus* as a function of total Zn soil concentrations after 2 weeks exposure to 30 nm ZnO, 200 nm ZnO and $ZnCl_2$ in four different soils. Each data point is the mean of three replicate samples. (•) Soil 1 - pH 4.5; (□) Soil 2 - pH 6.2; (Δ) Soil 3 - pH 7.3; and (X) Lufa 2.2 soil - pH 5.1.

3.5 Discussion

3.5.1 Soil characteristics

Soils spiked with 30 nm ZnO NPs and 200 nm ZnO showed similar characteristics in terms of soil pH and Ca^{2+} levels in the pore water. Also, zinc concentrations in the pore water were similar for both ZnO forms. The solubility of ZnO NPs has been shown to be very similar in comparison with 200 nm sized ZnO in Lufa 2.2 soil (Kool et al., 2011). For $ZnCl_2$ spiked soils, a different behavior was found (i.e. much higher Zn concentrations in the pore water were measured), as expected for a soluble metal salt. A decrease of Zn porewater concentrations with increasing soil pH was observed, while the Ca^{2+} concentrations in the pore water increased with increasing pH. The latter may be due to the fact that $CaCO_3$ was used to adjust soil pH. The addition of Zn^{2+} cations in the case of $ZnCl_2$ led to competition with protons and Ca^{2+} bound to the negatively charged soil particles, resulting in a decrease in soil pH and an increase in porewater Ca concentrations (Tipping et al., 2003). Zinc solubility is affected by soil pH, which is well described by the competitive adsorption model (Sauvé et al., 2000). In line with this, the competition between Zn^{2+} and Ca^{2+} ions in our tests resulted in an increase of Ca concentrations in solution (Figure 3.1), and the presence of protons and cations also affected zinc partitioning in soils.

The addition of nanoparticles resulted in either decreasing or increasing soil pH, depending on the nature of the soil. This probably is related to the buffer capacity of the soil as well as the nature of the particles. ZnO seems to increase rather than decrease soil pH, which may be related to its chemistry. The nanoparticle surface charge changes depending on the pH of the surrounding medium. The particles can reach the 'point of zero charge' (pHpzc) in which the positive and negative charges of the nanoparticles are equally balanced (Hassellöv et al., 2008). The dissolution reaction of Zn^{2+} ions from the NPs will consume protons and increase soil pH. But most ZnO particles will not dissolute and other reactions will take place on particle surfaces, more specifically on ZnOH groups that can undergo two reactions, depending on (porewater) pH. Below the pHpzc, the nanoparticle surface will adsorb protons, giving rise to a net positive surface charge and increasing pH. Above pHpzc, a second reaction dominates in which the surface

will release protons, gives rise to a net negative surface charge and acidify the soil.

This explains why pH increased in acid/neutral soil (pH 4.5-6.2) and slightly decreased at more basic soil (pH 7.3). In practice, however, it is more complex than that, because the change in pH brought about by adding the oxide will itself modify the oxide surface charge. So the pH at which the effect of the oxide switches from increasing the soil pH to decreasing it is not necessarily exactly the same as the pH of the point of zero charge.

A similar pH increase upon ZnO addition was also seen in Lufa 2.2 soil by Waalewijn-Kool et al. (2013a), but they observed a decrease later on when equilibrating the soils for up to one year. The reason for that pH decrease remains unclear, but it might be simply due to soil microbial activity.

ZnO nanoparticles were found to have a pHpzc above 7.5 in water (Kosmulski, 2004; Zhou and Keller, 2010) and above 8 in soil (Collins et al., 2012). At the highest pH_{CaCl2} of 7.3 in soil 3, i.e., closer to pHpzc, the attraction between NPs was increased, and consequently a greater diameter size would be expected. Heggelund and co-workers (2014) performing Transmission Electron Microscopy (TEM) analysis on $CaCO_3$ amended Dorset soils spiked with 30 nm ZnO, however, did not find an effect of soil pH on NP aggregate size. Dynamic Light Scattering (DLS) analysis showed that zeta potential was close to neutral for all soils, which was caused by the binding of organic matter to the NP surface, neutralizing the charge (Heggelund et al., 2014).

3.5.2 Toxicity to *Porcellionides pruinosus*

In this study, LC50 values for total Zn concentration in soil were comparable for all three Zn forms, with the exception of soil 1, where ZnO NPs were less toxic. ZnO NPs (>100 nm) and $ZnCl_2$ had no significant effect on the survival of the isopod *Porcellio scaber* exposed for 28 days via contaminated food in concentrations up to 5,000 mg/kg (Pipan-Tkalec et al., 2010). For the earthworm *Eisenia fetida*, $ZnCl_2$ had greater effect on survival when compared to 30 and 200 nm ZnO (Heggelund et al., 2014). For the springtail *Folsomia candida*, 30 nm and 200 nm ZnO had no effect on survival after 28 days exposure to up to 6,400 mg Zn/kg in

Lufa 2.2 soil (Kool et al., 2011). For $ZnCl_2$, the authors found an LC50 value of 1,000 mg Zn/kg. It therefore seems that isopods responded differently to ZnO and $ZnCl_2$ than other soil invertebrates, with less difference in sensitivity to the different Zn forms. This also means that for isopods, contrary to other soil invertebrates, particles are not less toxic than ionic Zn. This could also mean that particulate Zn contributes more to the toxicity of ZnO NPs or non-nano ZnO than the free Zn ions. Whether this is due to a fast dissolution of particulate Zn in the isopod's intestinal tract leading to an increased exposure to free Zn ions or a direct effect of the particles cannot be concluded from these data.

Zinc exposure induced a decrease of the biomass of the isopods independent of the Zn form present. Effects of $ZnCl_2$ on growth (mg/week) of the isopod *P. scaber* exposed via contaminated food have been reported in the literature. The EC50 value found by van Straalen et al. (2005) of around 30 µmol/g (corresponding to 1,980 mg Zn/kg dry food), was closely related to EC50 found by Donker et al. (1998) of about 33 µmol/g (corresponding to 2,230 mg Zn/kg dry food). These values are higher than the values found in this study for effects on biomass change, suggesting that soil exposure is more effective in reducing isopod growth when compared to food exposure. But it in fact is hard to compare both routes of exposure.

In this study, EC50 values reached the highest values at intermediate pH of approximately 6.0. The lowest EC50 value was found in soil 1 (pH_{CaCl2} 4.5). Even at the lowest concentration (i.e., 250 mg Zn/kg), the isopods showed a drastic decrease in biomass when exposed to soil 1 for all Zn forms, which could be due to a physiological response of the animals to the low soil pH. Litter acidification showed to decrease microbial density on leaf material (Zimmer et al., 2003), while the optimal microbial colonization was found at pH 5.0 (see references in (Zimmer et al., 2003)). Moreover, growth of the isopod *P. scaber* was influenced by leaf litter-colonizing microbiota when fed on alder leaf (Zimmer et al., 2003). Although we have no data on microbial communities in the tested soils, the trend observed seemed to be more related to a physiological effect on the biomass. It was observed for isopods that the preference for soil pH was species-specific (n=5) and that the preference ranged from pH 5 to 7 (Soejono Sastrodihardjo and van

Straalen, 1993). At present, no data on pH preference of *P. pruinosus* is available under laboratory conditions, making it hard to draw firm conclusions on the reason why ZnO and $ZnCl_2$ toxicity was lowest at the intermediate soil pH. Moreover, potential confounding factors may influence the results found. As indicated above, when increasing soil pH by adding $CaCO_3$, the soil solution will contain less H^+, but at the same time, higher Ca^{2+} levels and an increased Zn solubility are found. So, effects of pH may be confounded by the changes in the ionic strength in soil solution caused by pH adjustment.

The competition of Zn with Ca may have resulted in lower toxicity for $ZnCl_2$ than for ZnO based on porewater concentrations. In terrestrial crustaceans, calcium plays an important role, especially in the formation of the exoskeleton (Greenaway, 1985). They can absorb calcium either from food or from the cuticle itself (i.e., exuviae) (Greenaway, 1985). During the pre-molting period, the calcium from the old cuticle is transported and stored as $CaCO_3$ deposits, until reuse to form a new cuticle (Steel, 1993). However, in most soils, calcium is generally available in sufficient levels for isopods (Zimmer et al., 2000). In terms of toxicity, Ca^{2+} may have a protective effect by competing with Zn^{2+} in soil solution, decreasing metal toxicity, according to the biotic ligand model (BLM) (Spurgeon et al., 2006). In accordance with the BLM, the free concentration of metal ions and other cations in soil solution, combined with their competition to bind to the receptors on the organisms, will be the factors driving toxicity (Lofts et al., 2013; Plette et al., 1999; Thakali et al., 2006). The BLM considers the free metal ion to be the main metal form being available for uptake and causing effects, with other cations reducing toxicity by competing for the same uptake sites. When the activities of different cations in the soil pore water are known, the BLM can help to explain the toxicity and uptake of metals. The model therefore can be applied to environmental risk assessment studies, enabling comparison of soils with different characteristics. However, one should have in mind that not all organisms are equally exposed to soil solution, and that other routes of exposure might also be important. Considering the two main routes of chemicals to soil invertebrates (oral and dermal via), dermal uptake is less important for organisms with exoskeletons when compared to the oral uptake via the pore water (van Gestel and van

Straalen, 1994). Exchange of ions with the surrounding medium occurs through the uropods, located on the ventral abdomen. Uptake of ionic zinc (65 $ZnCl_2$) in *P. scaber* was shown to be similar in food (gut route) and soil exposures (gut and pleopod routes), indicating a low contribution of the pleopods as an uptake route (Vijver et al., 2006). For oral uptake, it is hard to distinguish the contribution of porewater and soil particles. Isopods mainly obtain water needs from the diet, but they can also absorb water from humid surfaces (Peijnenburg et al., 2012). From our data is seems that porewater concentrations do affect toxicity, at least when considering the EC50 values, but that other routes of exposure cannot be excluded.

For the collembolan *F. candida*, which is mainly exposed through the soil pore water (Smit and Van Gestel, 1996), toxicity decreased with increasing soil pH for all Zn forms (30 nm and 200 nm ZnO, and $ZnCl_2$), and EC50 for effects on reproduction was significant lower for $ZnCl_2$ than for ZnO particles (Waalewijn-Kool et al., 2013b). However, when EC50 values were based on Zn in pore water or free Zn^{2+} ion concentrations, the authors found that EC50 for $ZnCl_2$ was higher than for ZnO particles. The results were attributed to the protective effect of calcium, competing with Zn^{2+} ions and reducing toxicity (once calcium levels in pore water were much higher for $ZnCl_2$ spiked soils), combined with the decrease in pH values of $ZnCl_2$ spiked soils, which resulted in competition between Zn^{2+} and H^+ ions (Waalewijn-Kool et al., 2013b).

Differences between LC/EC50 based on Zn concentration in soil and pore water were found as well for survival and reproduction of *E. fetida* (Heggelund et al., 2014). In general, LC/EC50 values based on total concentration in soil were higher for ZnO particles than for $ZnCl_2$, however when based on porewater concentrations, ZnO particles showed lower values than $ZnCl_2$. The results provided by Waalewijn-Kool et al. (2013b) and Heggelund et al. (2014) are in accordance with the present results. It is suggested that such differences in EC50s between total soil concentrations and porewater concentrations might suggest a particle effect (Heggelund et al., 2014; Waalewijn-Kool et al., 2013b) but it possibly could also be explained from a protective effect of calcium in $ZnCl_2$ exposures following the principles of the Biotic Ligand Models (Waalewijn-Kool et al., 2013b).

The EC50 values for both ZnO NPs and ionic Zn found for the isopods in the present study were much lower than the values found by Waalewijn-Kool et al. (2013b) for the collembolans, possibly due to the dual possible uptake routes by soil ingestion and pore water.

The effect of pH on the bioavailability of metals is less pronounced for organisms with complex uptake routes (e.g. ingestion of soil particles) when compared to soil solution exposure (Plette et al., 1999). Vijver et al. (2006) showed that the main route of exposure to ionic zinc (as $ZnCl_2$) for the isopod *P. scaber* was the ingestion of contaminated soil particles (i.e., oral via). In the case of NPs, an even more complex scenario could be expected, as not only dissolution into ions, but also aggregation/agglomeration of nanoparticles will occur.

Zinc body content

Zinc body content was found to increase in a dose-dependent way in isopods exposed to 30 nm ZnO, 200 nm ZnO and $ZnCl_2$ in all tested soils. The increase in internal concentration indicates that the animals are storing zinc. The hepatopancreas of isopods is composed of S (small) and B (big) cells which functions are absorption and absorption/secretion, respectively (Hames and Hopkin, 1991b). While B cells secrete granules containing metals into the hepatopancreas tubules to be excreted through the faeces, S cells will accumulate the metals (Hopkin, 1990a). It has been shown that zinc will form granules in both B and S cells of *P. scaber* (Hames and Hopkin, 1991b). However, the capacity of excreting metals is species-dependent (Hames and Hopkin, 1991a).

Effects of pH on Zn body content were not observed when analyzing the data by ANOVA. The only significant difference for $ZnCl_2$ was found to be between soil 1 (pH_{CaCl2} 4.5) and Lufa soil (pH_{CaCl2} 5.5), indicating that other soil properties than soil pH may also be responsible for the difference. Similarly, soil pH did not affect ionic Zn accumulation in the earthworm *L. rubellus* (Spurgeon et al., 2006). However, for *E. fetida*, the bioaccumulation factor (BAF) was lower at higher pH of 7.3 (e.g., soil 3) for all Zn forms (30 and 200 nm ZnO and ionic zinc) than at lower pH (Heggelund et al., 2014). The absence of a clear pH effect might suggest soil ingestion is of importance as route of exposure in isopods.

Reductions in growth or reproduction occur when energy must be diverted to detoxification processes (Hopkin, 1990a). Effects of zinc on isopod growth are rather dependent on the fluxes of zinc between pools, being divided into an active pool and a storage pool (van Straalen et al., 2005). Metabolic processes in the active pool transfer zinc from the active to the storage pool, until a limit at which storage is no longer possible and free zinc ions can cause damage to the animal, resulting in growth reduction (van Straalen et al., 2005). So, no relation could be found between growth of isopods and zinc content in the hepatopancreas, once growth reduction is dependent on the fluxes of zinc between the pools rather than total Zn body content (van Straalen et al., 2005). Mortality, however, could be related to zinc concentration in the hepatopancreas (van Straalen et al., 2005).

Our results are in agreement with these findings. No difference in mortality and zinc body content between soils was found for all zinc forms. Growth however was affected by soil pH, and could not be related to zinc body content. As Zn body content did not differ between different soil pH levels, it may indicate that the isopods were able to store similar Zn quantities, but yet the effects (EC50) occurred at different soil concentrations. Zinc body content therefore could not predict sublethal toxicity, being in agreement with previous studies with growth rate of *P. scaber* exposed to $ZnCl_2$-contaminated food (Donker et al., 1998; van Straalen et al., 2005). In the earthworm *Eisenia veneta*, $ZnCl_2$ was found to be more toxic than ZnO NPs (e.g., reproduction and immune activity), however Zn body content was comparable between the two Zn forms (Hooper et al., 2011). Similarly, ZnO (30 and 200 nm) had greater Zn uptake compared to ionic Zn in *E. fetida*, however ionic Zn was more toxic (Loureiro et al, 2009). The relation between sublethal effects and Zn uptake is more complex for ZnO nanoparticles than for ionic zinc (Heggelund et al., 2014; Hooper et al., 2011).

Critical body concentration was found to be 25 g Zn/kg in the hepatopancreas of *P. scaber* before causing death by poisoning (Hopkin, 1990b). Van Straalen et al. (2005) transformed this data and found an equivalent critical total body concentration of 1,660 mg Zn/kg dry weight. This critical value is comparable to the maximum Zn levels found in *P. pruinosus* in this study (Figure 3.3).

Although zinc body content was found to be slightly higher in animals exposed to $ZnCl_2$, the levels were quite comparable to ZnO particles. Likewise, *P. scaber* feeding on ZnO NPs and $ZnCl_2$ contaminated food showed no differences in zinc body content, which was dose-dependent (Pipan-Tkalec et al., 2010). The authors concluded that the isopods accumulated zinc in the same manner for both zinc forms. In the case of soil exposure, even though the distributions of NPs and ionic zinc are completely different in the soil matrix, Zn accumulation in the isopods was similar independent of the Zn form (ZnO and $ZnCl_2$).

3.6 Conclusion

This study showed that soil pH did affect the toxicity of ZnO NPs, non-nano ZnO and $ZnCl_2$ to the isopod *P. pruinosus* in an indifferent way, with in general lowest toxicity at intermediate soil pH. Zn uptake seemed not to be affected by soil pH. There was little difference in Zn toxicity and Zn uptake between the different Zn forms, suggesting either a role of particulate ZnO in toxicity or a different contribution of routes of exposure, dependent on the Zn form. It seems oral ingestion may contribute more to uptake and effects of particulate ZnO, while toxicity of ionic Zn will also be influenced by properties of the pore water.

3.7 Acknowledgement

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3.9 Supplementary material

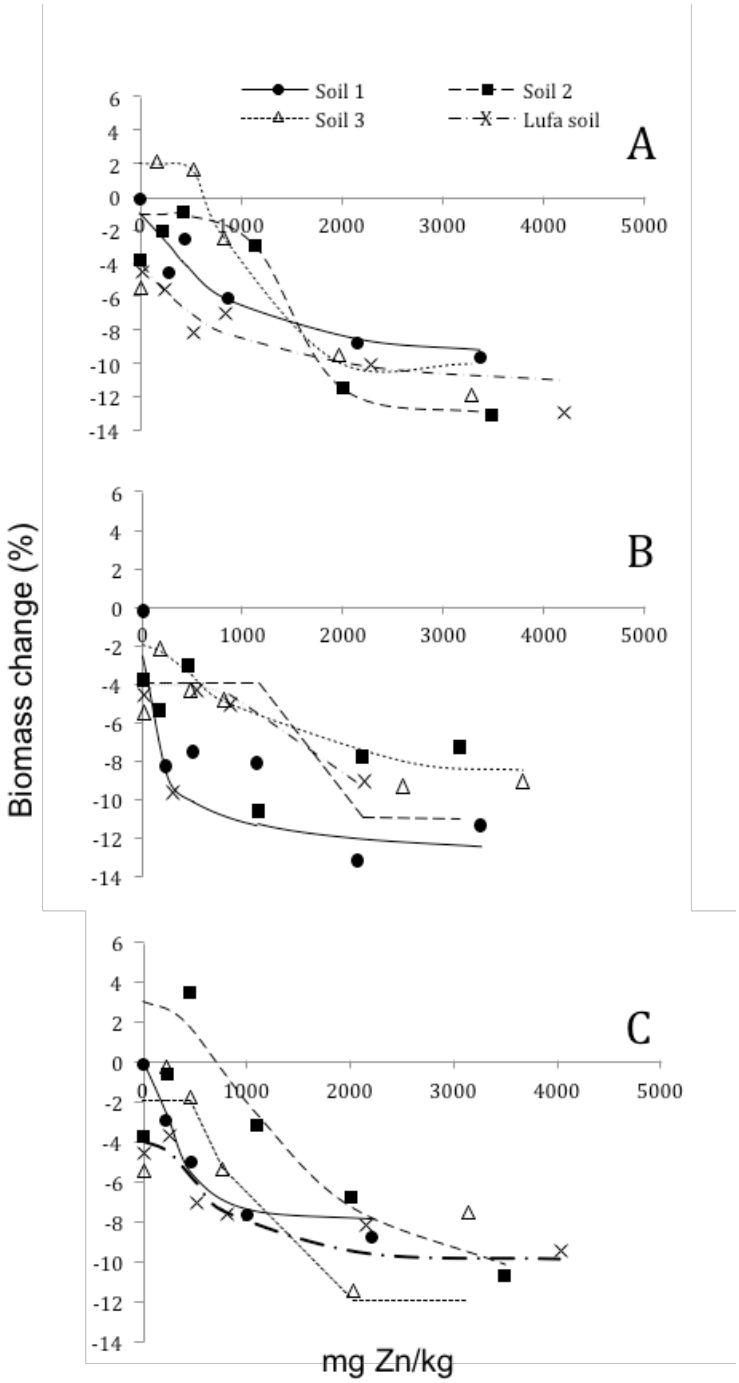

Figure S 3-1: Effects of 30 nm ZnO NPs, 200 nm ZnO, and $ZnCl_2$ on biomass change of the isopod *Porcellionides pruinosus* after 14 days exposure in four different soils (see Table 1). Lines represent the fit obtained with a logistic model for the different soils.

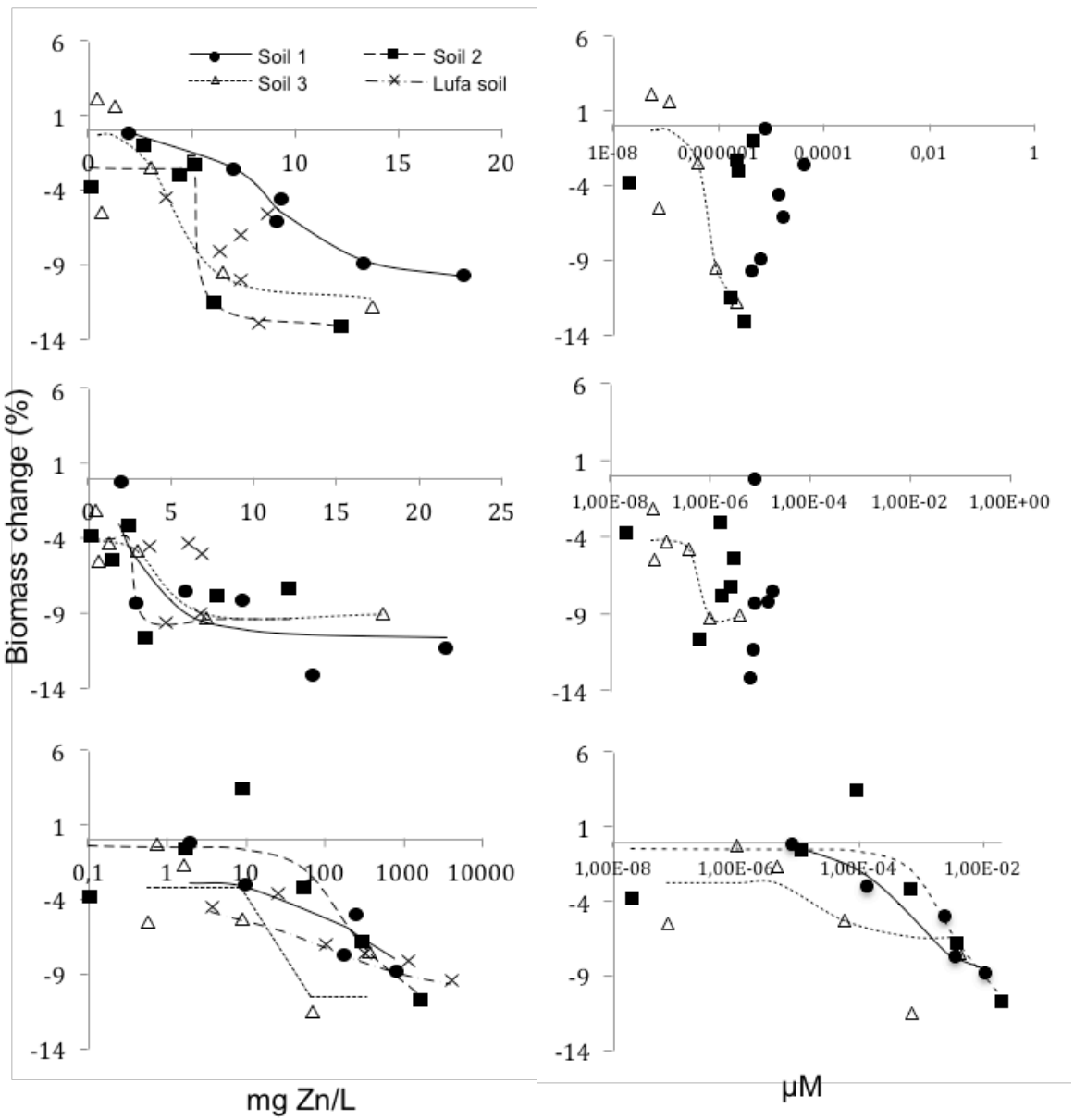

Figure S 3-2: Effects of 30 nm ZnO NPs, 200 nm ZnO, and $ZnCl_2$ on biomass change of the isopod *Porcellionides pruinosus* after 14 days exposure in four different soils (see Table 1). Biomass change is related to Zn concentrations in porewater (left) and free zinc ion concentrations calculated by WHAM7 (right) (plot in log scale for $ZnCl_2$) Lines represent the fit obtained with a logistic model for the different soil.

Table S 3.1: Measured total soil and porewater Zn concentrations in four different soils spiked with ZnO NPs (30 nm), non-nano ZnO (200 nm) and $ZnCl_2$ for determining the toxicity to *Porcellionides pruinosus*. Also included is pH of the pore water.

	Nominal concentration (mg Zn/kg)	Measured concentration (mg Zn/kg)				Porewater concentration (mg Zn/L)				Porewater pH			
		Soil 1	Soil 2	Soil 3	Lufa 2.2	Soil 1	Soil 2	Soil 3	Lufa 2.2	Soil 1	Soil 2	Soil 3	Lufa 2.2
	Control	4.2	4.0	4.4	11.8	1.93	0.10	0.57	3.68	5.09	6.69	7.03	6.22
30 nm ZnO	250	281	219	169	243	9.29	5.11	0.37	8.61	4.94	6.65	6.95	6.39
	500	451	425	518	532	7.01	2.62	1.23	6.35	5.55	6.59	7.25	6.48
	1000	871	1,142	828	848	9.11	4.39	2.96	7.37	5.80	6.89	7.14	7.35
	2000	2,161	2,011	1,961	2,285	13.3	6.00	6.47	7.37	6.51	7.26	7.25	7.70
	4000	3,369	3,492	3,279	4,196	18.1	12.1	13.7	8.21	7.14	7.35	7.43	6.5
200 nm ZnO	250	239	168	173	293	2.85	1.39	0.37	4.64	5.38	6.15	6.73	6.61
	500	503	453	470	531	5.82	2.41	1.16	5.99	5.42	6.51	7.07	6.74
	1000	1,138	1,153	805	866	9.23	3.36	2.9	6.78	5.79	6.95	7.10	7.41
	2000	2,142	2,193	2,595	2,201	13.5	7.75	7.10	6.75	6.55	6.97	7.26	7.74
	4000	3,369	3,160	3,780	4,542	21.6	12.1	17.8	8.28	6.94	7.19	7.35	7.73
$ZnCl_2$	250	228	244	221	256	9.72	1.66	0.74	25.6	4.47	6.07	7.1	5.5
	500	467	457	457	524	254	8.98	1.59	105.5	4.34	6.04	6.94	5.26
	1000	1,016	1,099	768	805	178	54.6	8.88	320	4.12	5.56	6.77	5.1
	2000	2,221	2,011	2,031	2,154	803	300	70.8	1,147	3.81	5.20	6.79	4.85
	4000	3,601	3,499	3,136	4,034	2,890	1,660	370	4,100	3.64	4.91	6.51	4.60

CHAPTER 4: EFFECTS OF SOIL AND DIETARY EXPOSURES TO Ag NANOPARTICLES AND $AgNO_3$ IN THE TERRESTRIAL ISOPOD *Porcellionides pruinosus*

Paula S. Tourinho[1], Cornelis A.M. van Gestel[2], Kerstin Jurkschat[3], Amadeu M.V.M. Soares[1], Susana Loureiro[1]

[1]Department of Biology and the Centre for Environmental and Marine Studies, University of Aveiro, Aveiro, Portugal

[2]Department of Ecological Science, Faculty of Earth and Life Sciences, VU University, Amsterdam, The Netherlands

[3]Department of Materials, Oxford University, Begbroke Science Park, Begbroke Hill, Yarnton, Oxford, OX5 1PF, United Kingdom

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4.1 Abstract

The effects of Ag NPs and $AgNO_3$ on the isopod *Porcellionides pruinosus* were determined upon soil and dietary exposures. Isopods avoided Ag in soil, with EC50 values of ~16.0 and 14.0 mg Ag/kg for Ag NPs and $AgNO_3$, respectively. Feeding inhibition tests in soil showed EC50s for effects on consumption ratio of 127 and 56.7 mg Ag/kg, respectively. Although similar EC50s for effects on biomass were observed for nanoparticulate and ionic Ag (114 and 120 mg Ag/kg dry soil, respectively), at higher concentrations greater biomass loss was found for $AgNO_3$. Upon dietary exposure, $AgNO_3$ was more toxic, with EC50 for effects on biomass change being >1500 and 233 mg Ag/kg for Ag NPs and $AgNO_3$, respectively. The difference in toxicity between Ag NPs and $AgNO_3$ could not be explained from Ag body concentrations. This suggests that the relation between toxicity and bioavailability of Ag NPs differs from that of ionic Ag in soils.

Keywords: toxicity; routes of exposure; silver nanoparticles; bioavailability

4.2 Introduction

Silver nanoparticles (Ag NPs) are widely used in the nanotechnology industry and consumer products, especially due to their bactericidal properties. The production of Ag NPs has increased in the last years, and it is one of the most produced NPs on the market (Meyer et al., 2009).

Due to the release from NP-containing products, Ag NPs may enter the aquatic environment and reach the soil through the land application of treated sewage sludge or biosolids (Gottschalk et al., 2009; Kaegi et al., 2011). In sewage sludge, the concentration was estimated to reach ~1.7 mg Ag/kg in Europe (Gottschalk et al., 2009). In biosolids-amended soils, however, much lower concentrations are expected, although the continuous input of biosolids to agricultural land may lead to increasing Ag-NP concentrations over time. For example, an increase rate of 36 µg Ag/kg/y is estimated for agricultural land through sludge application in the United Kingdom (Whiteley et al., 2013). Modeled Ag-NP concentrations in soils exclusively treated with sludge ranged from 10 to 100 µg/kg in Europe (Gottschalk et al., 2013). However, in sludge-free soils, estimated Ag-NP concentrations were 0.1-1 µg/kg (Gottschalk et al., 2013).

Toxicity studies on Ag NPs in soil have shown that responses may occur at low levels of exposure. In a field experiment, effects of Ag NPs on plants and microbial processes were found at concentrations as low as 0.14 mg Ag/kg (Colman et al., 2013). And avoidance behavior of earthworms, measured as EC50, has been reported at ~4-8 mg Ag/kg in natural soil (Shoults-Wilson et al., 2011b).

Up to date, dietary toxicity of NPs to isopods has previously been tested for Cu NPs (Golobič et al., 2012), TiO_2 NPs (Valant et al., 2012), ZnO-NPs (Pipan-Tkalec et al., 2010), and Ag NPs (Tkalec et al., 2011). Only two studies have evaluated the effects of soil exposure on isopods for ZnO and CeO_2 NPs (Chapter 3 and 6). Previous studies with the isopod *Porcellionides pruinosus* have shown that different routes of exposure (i.e. food and soil) need to be evaluated to properly assess the effects of contaminants on isopods (Sousa et al., 2000; Vink et al., 1995).

The objective of this study was to evaluate the toxicity of Ag NPs and ionic Ag to the terrestrial isopod *P. pruinosus*, using soil and food as exposure routes. For this

purpose, avoidance behavior and feeding inhibition were evaluated in natural soil spiked with Ag NPs and ionic Ag ($AgNO_3$). Additionally, a feeding inhibition test was conducted with Ag-dosed food, in order to assess the effects of Ag NPs and $AgNO_3$ upon dietary exposure and compare with soil exposure.

4.3 Methodology

4.3.1 Test organisms

Specimens of the isopod *Porcellionides pruinosus* were collected from a horse manure heap in an uncontaminated field (Coimbra, Portugal). The animals were kept in the lab at 20±2ºC and a 16/8h photoperiod for at least one month before use in the tests. For the toxicity tests, healthy adult males and non-gravid females (>15-25 mg) were used. Animals without antenna were not used, as their chemoreceptor organs are located in the apical organ of their second antenna, which can perceive chemicals and test stimuli.

4.3.2 Exposure media and test chemical

Lufa 2.2 soil (LUFA-Speyer 2.2, Sp 2121, LUFA Speyer, Speyer, Germany) and alder (*Alnus glutinosa*) leaves were used for the exposures. Lufa 2.2 is a loamy sand soil with pH (0.01 M $CaCl_2$) 5.5±0.2, water holding capacity (WHC) 41.8±3.0 %, 1.77±0.2 % organic C, 0.17±0.02 % nitrogen, 7.3±1.2 % clay; 13.8±2.7 % silt and 78.9±3.5 % sand. Alder leaves were collected from an uncontaminated area in Coimbra (Portugal) and stored at room temperature.

Soil and leaves were spiked with Ag NPs (AMEPOX, 3-8 nm, alkane coating) dispersed in pure water at 1,000 mg/L or $AgNO_3$ (Sigma-Aldrich, 99% purity) also dissolved in water. Result of Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) analyses of the Ag NPs can be found in Figures S 4-1 and S 4-2, respectively. Soils were moistened to 45% of the WHC, and left to equilibrate for one day before use in the toxicity tests.

4.3.3 Avoidance behavior test

Lufa 2.2 soil was spiked with both Ag forms at nominal concentrations of 1, 5, 10, 50, and 100 mg Ag/kg, while for Ag NPs also 500 mg Ag/kg was tested. Plastic boxes (135 x 85 mm) were divided in two compartments using a removable plastic split, where 40 g of moist Ag-spiked soil were added in one side and 40 g unspiked soil were added in the opposite side of the box (for details, see Loureiro et al. (2005)). The split was removed and 3 isopods were introduced. Three replicates were used for each concentration. Avoidance behavior was visually observed after 2h to check if an immediate response could be seen. After 48 hours, the split was reintroduced and the number of animals counted in each compartment (spiked or unspiked soil). The test also included a control having unspiked soil in both compartments.

4.3.4 Feeding inhibition test – soil exposure

Lufa 2.2 soil was spiked at nominal concentrations of 50, 100, 200, 400, and 800 mg Ag/kg for Ag NPs and 12.5, 25, 50, 100, and 200 mg Ag/kg for $AgNO_3$. These concentrations were based on a preliminary study, in which $AgNO_3$ showed high mortality at concentrations above 200 mg Ag/kg.

Isopods were weighted and placed individually in plastic boxes (Ø 65 mm), containing 20 g of moist soil. Five replicates were used for each concentration and control (unspiked soil). The animals were fed *ad libitum* with pieces of alder leaves that were dried at 50ºC and weighted. After 14 days, isopods were left for 1 day without food to empty their guts. Isopods and remaining food were weighted.

4.3.5 Feeding inhibition test – dietary exposure

For food exposure, alder leaves were cut in pieces of ~50 mg (dry weight). Solutions of Ag NPs or $AgNO_3$ at different concentrations were topically added to the leaves' surface with a micropipette. Solution concentrations were chosen in order to add the same volume of solution (400 µL) to each leaf portion. Half of this volume was applied on each side of the leaves to guarantee a more homogeneous distribution of Ag on the surface. A control with ultrapure water was also included.

Leaves were dried at room temperature for one day before being offered to the isopods as food (*ad libitum*). The test vessel was composed of two plastic boxes (Ø 85 mm) placed inside each other (Loureiro et al. 2006). The inner box with a net in the bottom was used to easily collect the faeces and avoid coprophagy. The outer box had a bottom of plaster of Paris and was used to maintain high air humidity. Individual isopods and food were placed in the chambers, with 10 replicates per concentration. After 14 days exposure, the fresh weight of isopods and dry weight of remaining food and faeces were measured.

4.3.6 Chemical analysis

Soil pH was measured in 0.01 M $CaCl_2$ extracts of freshly spiked soils, in accordance to ISO guideline 10390 (ISO, 1994).

For total Ag analysis, single replicate samples of approximately 130 mg soil or 30 mg leaf material were dried overnight at 50ºC. The samples were then digested in 2 mL of a mixture of concentrated HCl (J.T. Baker, purity 37%) and HNO_3 (J.T. Baker, purity 70%) (4:1, v/v) for 7 hours in an oven (CEM MDS 81-D) at 140ºC, using tightly closed Teflon containers. After digestion, the samples were taken up in 10 mL of demineralized water and analyzed by flame atomic absorption spectrometry (AAS) (Perkin-Elmer AAnalyst 100). Certified reference material (ISE sample 989 of River Clay from Wageningen, The Netherlands) was used to ensure the accuracy of the analytical procedure. Ag concentration in the reference material (mean±SE; n=2) was 125±1.4% of the certified value.

Total Ag concentrations were also determined in the isopods. After freeze-drying, three isopods from each treatment were individually weighted and digested in 300 µL of a mixture of HNO_3:$HClO_4$ (7:1, v/v, J.T. Baker, ultrapure). The samples were evaporated to dryness and the residues were taken up in 1 mL 1M HCl. Silver content was determined by graphite furnace AAS (Perkin-Elmer 5100 PC).

4.3.7 TEM images

Transmission Electron Microscopy (TEM) analyses were performed on unspiked soil and on soil spiked with Ag NPs and $AgNO_3$ at 800 and 200 mg Ag/kg, respectively. Approximately 10 mg of soil were dispersed in 10 mL deionized

water, and sonicated in an ultrasonic bath for 30 seconds. Then, 20 µl of the dispersion was suspended on a carbon coated Cu TEM grid. TEM was carried out on a 200kV analytical JEOL 2010 instrument with an Oxford Instruments EDX detector. TEM micrographs were taken from several regions with small grains. Large grains are not electron transparent and were excluded from this analysis.

4.3.8 Data analysis

Avoidance response (%) was calculated as Loureiro et al. (2005):

$A=(C-T)/N*100$

where C is the number of animals in control soil, T is the number of animals in spiked soil and N is the total number of animals recovered from the soil. The median effect concentration (EC50) was calculated using a two-parameter logistic curve.

For the feeding inhibition test, the feeding parameters were calculated as:

$Cr = (W_{Li} - W_{Lf}) / W_{isop}$

$Ar = ((W_{Li} - W_{Lf}) - F) / W_{isop}$

$Ae = ((W_{Li} - W_{Lf}) - F) / (W_{Li} - W_{Lf}) * 100$

$Eg = F / W_{isop}$

$Bc = ((Wi_{sopf} - W_{isop}) / W_{isop}) * 100$

where, W_{Li}—initial leaf weight (mg d.w.); W_{Lf}—final leaf weight (mg d.w.); W_{isop}—initial isopod weight (mg f.w.); W_{isopf}—final isopod weight (mg f.w.); F—faeces (mg d.w.); Cr—Consumption ratio (mg leaf/mg isopod); Ar—assimilation ratio (mg leaf/mg isopod); Ae—assimilation efficiency (%); Eg—egestion ratio (mg faeces/mg isopod); Bc—biomass change (%).

For the feeding inhibition test in soil, consumption ratio was analyzed by one-way analysis of variance (ANOVA) after log-transformation. The median effect concentration (EC50) for the consumption ratio (mg food/mg isopod) and biomass change (% of fresh weight) was calculated with a four-parameter logistic regression. The Bioaccumulation factor (BAF) was calculated as the ratio between Ag concentration in the isopods and total Ag concentration in soil. For the feeding inhibition test upon dietary exposure, the feeding parameters were log transformed to achieve normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's

test) and analyzed by one-way ANOVA followed by Dunnett's post hoc test. If even after log transformation data failed normality or homoscedasticity tests, a Kruskal-Wallis one-way analysis of variance on ranks was conducted. When significant differences were found, a Dunn's post-hoc test was performed.

The relation between biomass change (%) and Ag body concentration in isopods was tested by Spearman correlation analysis, and the relation between Ag body concentration in isopods and Ag concentrations in soil/food by regression analysis. Normality of the residuals was tested by Kolmogorov-Smirnov test and homoscedasticity was graphically analyzed by plotting standardized residuals versus predicted values. Data was squared root transformed when necessary. Analysis of covariance (ANCOVA) was conducted to compare the slopes obtained in the regression analysis, with Ag form as independent variable, Ag body concentration as dependent variable and Ag concentration in soil/food as covariant. The homogeneity of slopes was tested prior to the analysis ($p<0.05$) and normality and homoscedasticity were tested with Kolmogorov-Smirnov and Levene's tests, respectively. For dietary exposure, ANCOVA analysis could not be performed, since assumptions were violated even after data linearization.

4.4 Results

4.4.1 Ag analysis in soil and food

Measured total Ag concentrations in the soils of the avoidance behavior and feeding inhibition tests are shown in Tables S 4-1 and S 4-2, respectively. In general, Ag recovery was satisfactory, ranging between 75 and 125 % of the nominal concentrations (Table S 4-1 and S 4-2). Recovery was lower at 800 mg Ag/kg (55%) for Ag NPs and at 50 and 100 mg Ag/kg (67-68%) for $AgNO_3$ in the feeding inhibition test, and higher at 1 mg/kg (310-320%) for both Ag forms and at 5 and 10 mg Ag/kg (170-187%) for $AgNO_3$ in the avoidance test.

Measured Ag concentrations in the leaves are shown in Table S 4-3. In most treatments, recovery was good for both Ag forms, ranging from 62 to 114%. However, at the highest nominal concentration of 3,000 mg Ag/kg, recovery was 50 and 39% for Ag NPs and $AgNO_3$, respectively.

All effect concentrations reported in this paper were calculated based on measured Ag concentrations in soil or food.

4.4.2 Soil properties

Soil pH showed little difference among Ag NPs spiked soils, ranging from 5.52 to 5.81 (Table S 4-2). For ionic Ag spiked soils, a slight decrease in soil pH was observed with increasing Ag concentration, from 5.57 at 12.5 mg Ag/kg to 5.33 at 200 mg Ag/kg (Table S 4-2).

TEM of unspiked Lufa 2.2 soil showed a low background of Ag (0.1-0.2%) (Figure S 4-3A). In soil spiked with Ag NPs at 800 mg Ag/kg, clusters of Ag particles could be found in many areas (Figure S 4-3B), which closely resemble the original Ag particles. A small number of larger particles could also be detected, ranging from 20 to 50 nm (Figure S 4-3C). At higher magnification, it was possible to identify individual Ag particles, with particle sizes ranging from 5 to 8 nm (Figure S 4-3D). TEM images of Lufa soil spiked with $AgNO_3$ can be found in Figure S 4-4, where no particles could be pinpointed.

4.4.3 Avoidance behavior test

No mortality of isopods was observed during the avoidance behavior test. The isopods were able to avoid both Ag NPs and ionic Ag in soil (Figure 4.1; Table 4.1). Based on the overlap of 95% confidence intervals, no difference in EC50 was observed between both Ag forms. A limit value of >80% of organisms located in the control soil is considered to indicate impairment of the habitat function of soils (Hund-Rinke and Wiechering, 2001). In the present study, >80% of the isopods were found in the control soil at 36 and 18 mg Ag/kg dry soil for Ag NPs and ionic Ag, respectively (Figure 4.1).

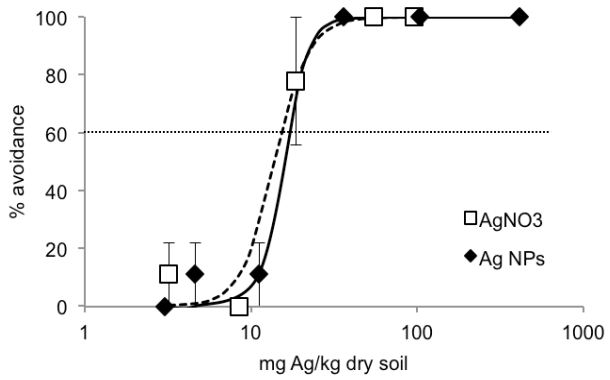

Figure 4.1: Percentage (mean±SE; n=3) of isopods (*Porcellionides pruinosus*) in control soil after 48h in the avoidance behavior test with Ag-NPs and ionic Ag ($AgNO_3$) on Lufa 2.2 soil. Solid (Ag-NPs) and dash ($AgNO_3$) lines represent the fit obtained with a 2-parameter logistic dose-response model. The dotted line represents the criterion for habitat function, where >80% of the organisms were located in the control soil (equal to 60% avoidance).

4.4.4 Feeding inhibition test – Soil exposure

No mortality was observed in isopods exposed to Ag NPs, while one out of five animals died in the ionic Ag exposure at 100 mg Ag/kg dry soil. Consumption ratio (mean±SE; n=5) was 0.38±0.05 and 0.30±0.06 mg food/mg isopod in control animals for the tests with Ag NPs and $AgNO_3$, respectively (Figure 4.2A). Consumption ratio was significantly decreased in isopods exposed at 153 and 252 mg Ag/kg dry soil for Ag NPs and ionic Ag, respectively (One-way ANOVA, Dunnett's post-hoc test). EC50s for effects on consumption ratio can be found in Table 4.1.

Table 4-1: LC50 for effects for Ag NPs and $AgNO_3$ on the survival, EC50 for effects on consumption ratio, biomass change and avoidance behavior of the isopod *Porcellionides pruinosus.* LC50 and EC50 values for effects on consumption ratio and biomass were obtained after 14d exposure to Ag-dosed Lufa 2.2 soil or alder leaves. EC50 values for avoidance behavior were calculated from the 48-h avoidance behavior test in Lufa 2.2 soil. 95% Confidence intervals are presented in between brackets.

	Soil exposure				Dietary exposure
	LC50	EC50 Consumption ratio	EC50 Biomass	EC50 Avoidance behavior	EC50 Biomass
Ag NPs	>455	127 (56.4 – 200)	114 -	15.8 (0.24 – 31.4)	>1500
$AgNO_3$	396[a] (235 - 745)	56.7 (8.33-105)	120 -	13.9 (10.1 – 17.7)	233 -

[a] Based on nominal concentrations.

Biomass change showed a dose-related decrease in isopods exposed to both Ag NPs and ionic Ag (Figure 4.2B). Even though EC50s for both Ag forms were similar (Table 4.1), biomass was strongly reduced by ionic Ag at higher concentrations. Biomass was reduced up to 8.2% at 361 mg Ag/kg for Ag NPs, while for ionic Ag, biomass reduction was up to 15.2% at 251 mg Ag/kg. In a pilot test, ionic Ag caused high isopod mortality at 400 and 800 mg Ag/kg dry soil, indicating it is more toxic than Ag NPs.

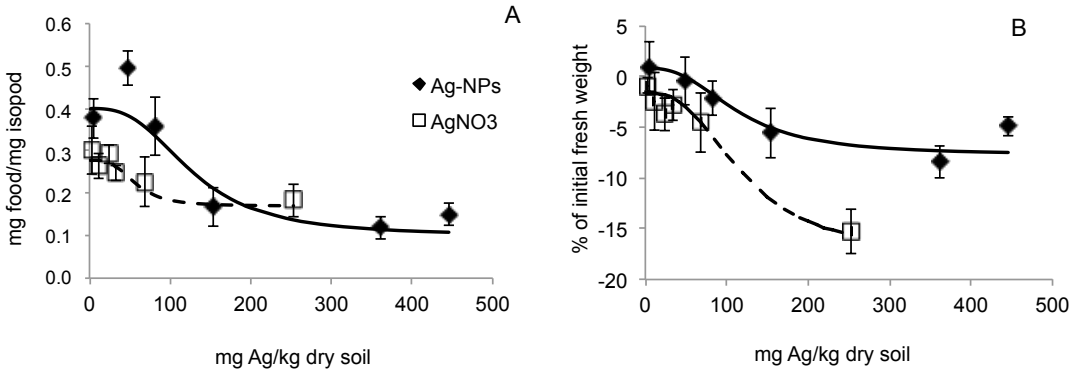

Figure 4.2: Effects of Ag NPs and ionic Ag ($AgNO_3$) on the consumption ratio (mean±SE; n=5) (A) and biomass change (mean±SE; n=5) (B) of the isopod *Porcellionides pruinosus* after 14 days exposure in Lufa 2.2 soil. Solid (Ag NPs) and dash ($AgNO_3$) lines represent the fit obtained with a 4-parameter logistic dose-response model.

Total Ag body concentration in isopods dose-related increased for both Ag forms (Figure 4.3). A significant linear relationship was found for Ag NPs (r2=0.77, p=0.00) and $AgNO_3$ (r2=0.72, p=0.00). No significant difference in the regression slopes was observed between Ag NPs and $AgNO_3$ (ANCOVA, F=1.62, p>0.05), but soil concentration significantly affected Ag body concentration (ANCOVA, F=81.5, p=0.00). Bioaccumulation factors (BAF) were similar for the two Ag forms and tended to decline with increasing exposure level (Table S 4-2).

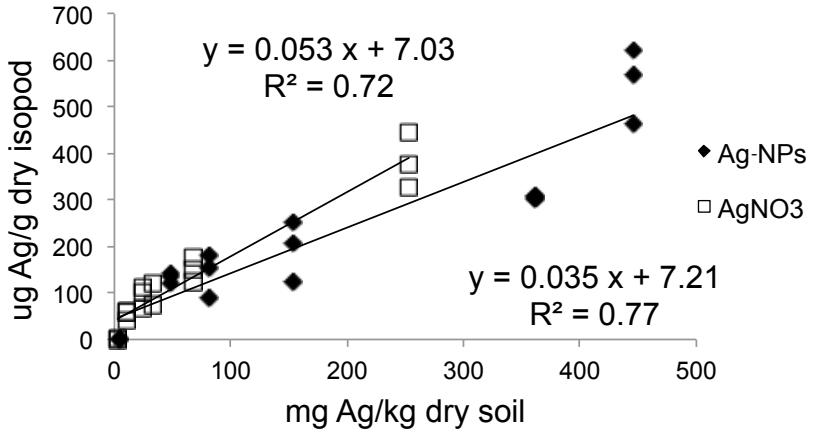

Figure 4.3: Linear regression between Ag body concentrations (µg Ag/g dry body weight) and total Ag concentrations in Lufa 2.2 soil (mg Ag/kg dw) in the isopod *Porcellionides pruinosus* after 14 days exposure to Ag-NPs (◆) and ionic Ag as $AgNO_3$ (□).

To link toxicity to Ag body concentration, effects on biomass were related to Ag in body using a correlation analysis (Figure 4.4). No significant relationship was found for Ag NPs (Spearman correlation, r= -0.16, p=0.51), while for ionic Ag a weak but significant relation was found (Spearman correlation, r=-0.65, p=0.00).

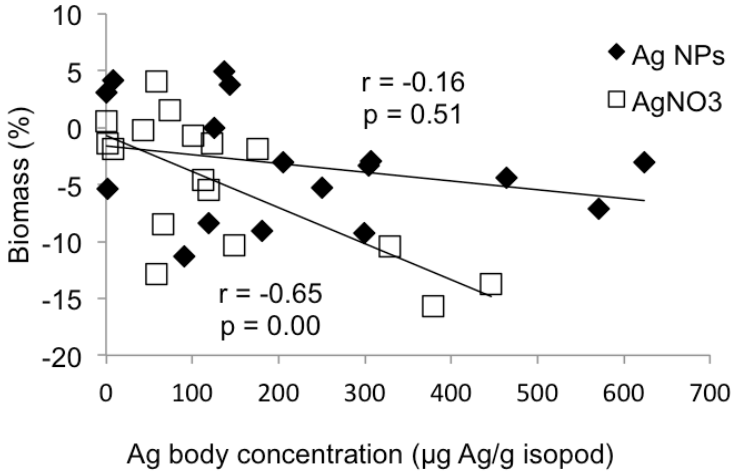

Figure 4.4: Biomass change (%) as a function of Ag body concentration (ug Ag/g dry body weight) in the isopod *Porcellionides pruinosus* exposed for 14 days to Ag NPs (◆) and ionic Ag (□) in Lufa 2.2 soil (n = 3 for each exposure concentration in soil). Spearman correlation coefficient (r) and p-values are given for Ag-NPs and $AgNO_3$.

4.4.5 Feeding inhibition test - Dietary exposure

No mortality was observed in control animals, while mortality rate was 20% (2 out of 10 isopods) for Ag-NP exposure at 114 mg Ag/kg dry food. For $AgNO_3$, mortality rate was 60% (6 out of 10 isopods) at 279 and 785 mg Ag/kg dry food, and 40% at 1159 mg Ag/kg dry food. Consumption ratio in the control (0.82 mg food/mg isopod) was significantly higher than for both Ag-NP (One-way ANOVA, $F=6.76$, $p<0.05$) and $AgNO_3$ exposure ($F=7.13$, $p<0.05$) (Figure 4.5A). Upon Ag-NP exposure, consumption ratio ranged from 0.35 to 0.49 mg food/mg isopod and was significantly lower than the control in all treatments (Dunnett's test, $p<0.05$). Upon $AgNO_3$ exposure, consumption ratio varied from 0.22 to 0.48 mg food/mg isopod, and was significantly different from the control for all treatments, except for 62 mg Ag/kg dry food (Dunnett's test, $p<0.05$). Assimilation ratio in control animals was 0.45 mg food/mg isopod, while it ranged between 0.25-0.42 and 0.18-0.38 mg food/mg isopod for the Ag-NP and $AgNO_3$ exposures, respectively (Figure 4.5B). Assimilation ratio decreased significantly in isopods exposed to Ag NPs (Kruskal-Wallis one-way ANOVA on ranks, $H=15.78$, $p<0.05$) and $AgNO_3$ ($H=12.98$, $p<0.05$), with significant differences at 29 mg Ag/kg dry food for Ag NPs, and at 22 and 279 mg Ag/kg dry food for $AgNO_3$ (Dunn's test, $p<0.05$). Assimilation

efficiency increased with increasing concentration for both Ag exposures (Figure 4.5C). However, significant differences were only found for $AgNO_3$ (Kruskal-Wallis one-way ANOVA on ranks, $H=13.02$, $p<0.05$), but not for Ag NPs ($H=9.54$, $p>0.05$). Assimilation efficiency was significantly higher at the highest concentration of $AgNO_3$ (92%) compared with the control (71%) (Dunn's test, $p<0.05$). In control animals, egestion ratio (0.30 mg faeces/mg isopod) was significantly higher than for Ag-NP (0.05-0.12 mg faeces/mg isopod) (Kruskal-Wallis one-way ANOVA, $H=17.21$, $p<0.05$) and $AgNO_3$ exposures (0.05-0.13 mg faeces/mg isopod) ($H=20.34$, $p<0.05$) (Figure 4.5D). Egestion ratio was significantly decreased at concentrations ≥218 mg Ag/kg dry food for Ag NPs, and at 279 and 1159 mg Ag/kg dry food for $AgNO_3$ (Dunn's test, $p<0.05$).

Biomass increased 3.16% in control animals after 14 days. Ag-NP exposure decreased biomass from 0.48 to -1.85%, but this decrease was not dose-related to Ag concentration in food (Figure 4.5E). No significant difference was found between Ag-NP and control treatments (Kruskal-Wallis one-way ANOVA, $H=8.32$, $p>0.05$). Upon $AgNO_3$ exposure, biomass significantly decreased with increasing Ag concentration in food ($H=14.59$, $p<0.05$). A significant difference was observed at the highest concentration (Dunn's test, $p<0.05$), with mean biomass change of -5.72%. For $AgNO_3$ exposures, EC50 was 233 mg Ag/kg (Figure 4.5E).

Ag body concentration tended to increase with increasing Ag concentration in food (Figure 4.6). A steeper slope was observed for $AgNO_3$ in comparison to Ag NPs. No significant relationship was found between biomass change and Ag body concentrations upon dietary exposure to both Ag forms (data not shown).

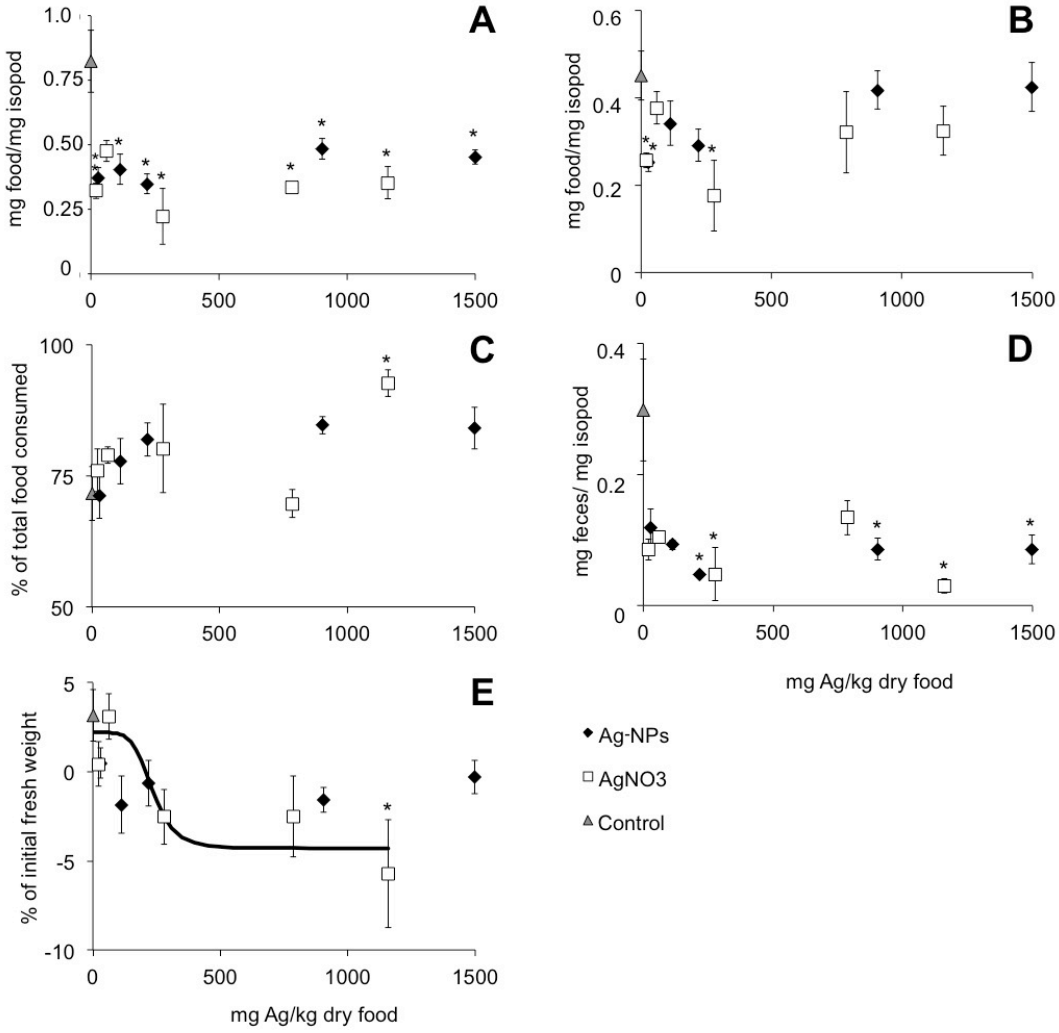

Figure 4.5: Effects on consumption ratio (A), assimilation ratio (B), assimilation efficiency (C), egestion ratio (D), and biomass change (E) of isopods (*Porcellionides pruinosus*) exposed for 14 days to Ag-NP and $AgNO_3$ dosed food (mg Ag/kg dry food). Line represents the fit obtained with a 4-parameter logistic dose-response model for effects of $AgNO_3$ on isopod biomass change.

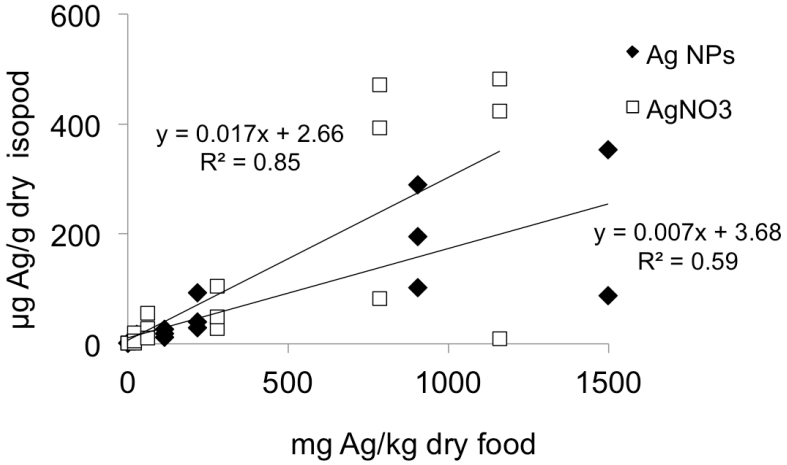

Figure 4.6: Ag body concentrations (µg Ag/g dry body weight) in the isopod *Porcellionides pruinosus* as a function of total measured Ag concentrations in food (mg Ag/kg dw) after 14 days exposure to Ag-NPs (◆) and ionic Ag as $AgNO_3$ (□). Lines represent the linear relationship between mean Ag body concentration and mean measured Ag concentration in soil.

4.5 Discussion

Here, isopods were exposed to Ag NPs and $AgNO_3$ via soil and food. When comparing the EC50 for effects on biomass change (Table 4-1), higher toxicity of both Ag NPs and $AgNO_3$ were found following soil exposures. These findings are in agreement with the study of Vink et al. (1995), who found higher LC50s for the same species exposed to three pesticides via food in comparison to substrate (e.g., sand).

In our study, the slopes of the relation between Ag body concentration and exposure concentration were 5 and 3 times higher for soil exposure than for dietary exposure for Ag NPs and $AgNO_3$, respectively. Similarly, Sousa et al. (2000) observed that lindane assimilation rate in *P. pruinosus* was up to 20 times higher in soil exposures. The lower bioavailability and toxicity upon dietary exposure was explained by the higher organic matter content of food, resulting in stronger binding of contaminants in comparison to soil (Sousa et al., 2000; Vijver et al., 2006; Vink et al., 1995).

4.5.1 Ag availability in soil

The toxicity of NPs will depend not only on their properties, but also on the processes occurring in soils, such as aggregation/agglomeration and dissolution. In the present study, Ag NPs were found as small aggregates of >50 nm and as single particles (5-8 nm) when spiked into the natural Lufa 2.2 soil (Figure S 4-3). Aggregation may affect dissolution rates, as dissolution was found to be slower for larger aggregates (Coutris et al., 2012). Dissolution of Ag ions from Ag NPs occurs through oxidation processes, which will depend on reactions on the particle surface (Batley et al., 2012; Shoults-Wilson et al., 2011a). However, low levels of oxidized Ag are normally reported in the literature (Cornelis et al., 2012; Cornelis et al., 2013; Coutris et al., 2012; Shoults-Wilson et al., 2011a; Waalewijn-Kool et al., 2014). Ag NPs may bind to soil particles (Coutris et al., 2012), especially to clays resulting in low Ag concentrations in pore water (Cornelis et al., 2012).

In agreement, only low Ag levels were found in the pore water of Lufa 2.2 soil spiked with Ag NPs and $AgNO_3$ (Waalewijn-Kool et al., 2014). The authors found that Ag porewater concentration was less than 1.5% of the total Ag concentration in soil. In the present study, similar conditions and the same Ag NPs were used as in the study by Waalewijn-Kool et al. (2014). Thus, Ag porewater concentrations in our study are also expected to be rather low in both Ag-NP and $AgNO_3$ spiked soils.

4.5.2 Avoidance behavior

The EC50 values for Ag NPs and ionic Ag indicated that isopods were able to detect and avoid Ag at relatively low soil concentrations, independent of the Ag form. As low levels of freely dissolved Ag are expected in soil pore water, we may conclude that not only Ag+ ions but also the nanoparticles were responsible for the avoidance behavior of the isopods.

Similar high sensitivity of avoidance responses was found for the earthworm *Eisenia fetida* with EC50s of 4.80, 8.74, and 6.06 mg Ag/kg dry soil for 10 nm Ag NPs, 30-50 nm Ag NPs, and $AgNO_3$, respectively (Shoults-Wilson et al., 2011b). Avoidance of Ag NPs was related to nanosized Ag, since the test duration (48h) was not long enough to expect much dissolution of Ag+ ions from the NPs

(Shoults-Wilson et al., 2011b). Moreover, these authors observed an immediate avoidance of $AgNO_3$ spiked soil (after 2h), but not of Ag-NP spiked soil, that could be due to the faster perception of Ag+ ions by the earthworms. In our study, no avoidance of ionic Ag by the isopods was observed after 2h of exposure, which could be explained from differences in exposure routes between isopods and earthworms. Earthworms not only have a soft body, but also live in close contact to soil, being dermally exposed to soil pore water (Van Gestel and Van Straalen, 1994). Isopods, on the other hand, have a hard body (cuticle), being less exposed to the dissolved ions in pore water (Van Gestel and Van Straalen, 1994).

4.5.3 Feeding inhibition – soil exposure

Ag NPs and ionic Ag exposures decreased food consumption and biomass in isopods exposed via soil for 14 days. At concentrations up to 100 mg Ag/kg in soil, biomass decreased in the same dose-related manner for both Ag forms, leading to these similar EC50 values (Figure 2B). However, at the higher exposure concentrations, ionic Ag showed to be much more toxic. A drastic decrease in the biomass was observed in isopods exposed to ionic Ag at >100 mg Ag/kg dry soil, while effects seemed to level off in isopods exposed to Ag NPs up to ~500 mg/kg dry soil. Moreover, a preliminary test with ionic Ag (concentrations up to 800 mg Ag/kg dry soil) resulted in high mortality, with an LC50 of 396 mg Ag/kg dry soil (nominal concentration, data not shown). For Ag NPs, no mortality was observed in the isopods exposed up to ~500 mg Ag/kg dry soil. Our results confirm the difference in toxicity between Ag NPs and ionic Ag. For instance, effects on biomass could not be related to Ag body concentration for Ag NPs, while for $AgNO_3$ biomass was negatively related with Ag body concentration.

Nevertheless, the higher toxicity observed for ionic Ag could not be explained from Ag bioaccumulation as no difference in Ag body concentration was found between Ag NPs and $AgNO_3$ when comparing the regression slopes. A lack in relationship between nanoparticle toxicity and body concentration was also observed in other studies with different nanoparticles. ZnO-NP toxicity, for instance, could not be directly related to Zn body concentration in earthworms (Heggelund et al., 2014) and isopods (Chapter 3).

Still, it is unclear whether toxicity in isopods exposed to Ag NPs is caused only by Ag ions dissolved from Ag NPs inside the body or by a combination of nanosized and ionic Ag. Dissolved Ag from NPs was responsible for toxicity in the earthworms *E. fetida* and *E. andrei* (Schlich et al., 2013; Tsyusko et al., 2012), the collembolan *Folsomia candida* (Waalewijn-Kool et al., 2013), and the nematode *Caenorhabditis elegans* (Meyer et al., 2010). Even though a particle effect was suggested, toxicity still was mainly related to ionic Ag for the potworm *Echytraeus albidus* (Gomes et al., 2013) and the earthworm *E. fetida* (Gomes et al., 2015).

4.5.4 Feeding inhibition - Dietary exposure

Isopods were able to detect and avoid food dosed with both Ag NPs and ionic Ag, by decreasing food consumption. Avoidance of highly contaminated food has been observed in *P. pruinosus*, and considered to be metal-specific (Loureiro et al., 2006). EC50s for the effect on consumption ratio were 10.3 and 11.1 mg/g dry food for Cu and Zn, respectively, while no decrease in food consumption was observed in isopods exposed to Cd and Pb (Loureiro et al., 2006). Effects on food consumption have been observed for the isopod *Porcellio scaber* exposed to food dosed with Zn (Bibič et al., 1997; Donker et al., 1996; Drobne and Hopkin, 1995; Zidar et al., 2003) and Cu (Farkas et al., 1996), and for the isopod *Oniscus asellus* exposed to Co (Drobne and Hopkin, 1994). In contrast to the present study, no decrease in food consumption was observed for the isopod *P. scaber* exposed for 14 days to Ag-NP dosed food (up to 5000 µg Ag/g dry food) (Tkalec et al., 2011). Probably, the avoidance of Ag-dosed food differs between isopod species, while it may also depend on the type of food, the way of spiking the food and the type of NPs (e.g. coating). The authors used hazelnut leaves as food and Ag NPs with a diameter size between 30-200 nm.

The avoidance of Ag-dosed food led to a decrease in assimilation and egestion ratios compared to the control, even though it did not decrease in a dose-related manner. Assimilation efficiency tended to increase with increasing Ag concentration in food. Isopods can increase assimilation efficiency by increasing the residence time of the food in the digestive tract, as a consequence of low quality or contaminated food (Drobne and Hopkin, 1994; Drobne and Hopkin,

1995). Overall, inhibition of feeding activity was observed when isopods were exposed via food to both Ag NPs and $AgNO_3$.

No significant decrease in biomass was observed in isopods exposed to Ag NPs up to ~1500 mg Ag/kg dry food. In agreement, no effect of Ag NPs on weight change was observed in the isopod *P. scaber* when exposed up to 5,000 mg/kg for 14 days (Tkalec et al., 2011). Nevertheless, not only did biomass significantly decrease, biomass loss was also higher in isopods exposed to $AgNO_3$-dosed food when compared to Ag NPs. Thus, toxic effects on biomass could only be observed for ionic Ag, but not for AgNPs, when exposed via food.

We used an indirect exposure by topically applying Ag as solution on the food. Indirect exposure was shown to lead to lower assimilation of Au-NPs in comparison to direct exposure (Judy et al., 2012; Unrine et al., 2012). One possible reason for that is the aggregation of NPs after the solution has dried on the leaf surface, decreasing NP bioavailability (Judy et al., 2012). If Ag-NP aggregation also increased due to the spiking procedure in our study, it could explain the slightly lower slope of the regression line for Ag body concentration in Ag-NP exposure.

4.6 Conclusions

Ag NPs and $AgNO_3$ affected the avoidance behavior and feeding activity in isopods exposed via soil and food. The isopod *Porcellionides pruinosus* can avoid low concentrations of Ag in soil, independent of the Ag form (i.e., nanosized or ionic Ag). Still, these concentrations were around two-fold higher than predicted Ag concentrations in soils amended with sewage sludge.

In the feeding trials for both soil and dietary exposures, Ag NPs were found generally to be less toxic than $AgNO_3$. Following soil exposure, ionic Ag caused greater biomass losses and mortality, while Ag NPs caused no mortality and had less effect on biomass. Ag body concentrations failed to explain these differences in toxicity, since Ag similar levels were found in isopods exposed to Ag NPs and ionic Ag. In agreement, upon dietary exposure, higher toxicity was found for ionic Ag, whereas no effects on the biomass could be observed in the Ag-NP treatments. These differences in toxicity between the two Ag forms, which need

further investigation, may be key to an appropriate risk management of Ag NPs in terrestrial environments.

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4.8 Supplementary material

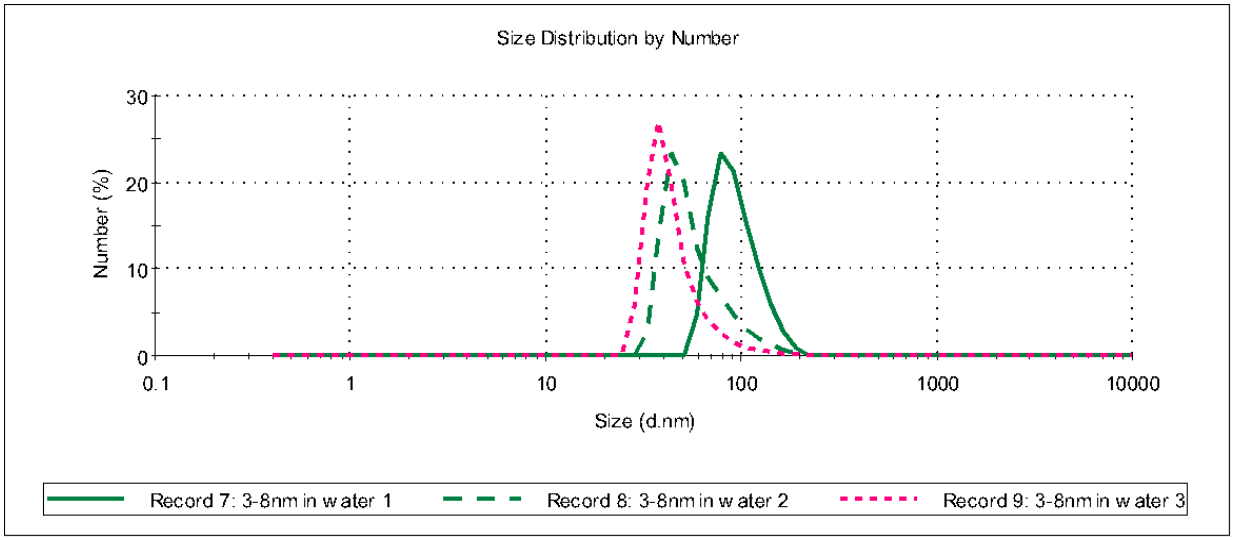

Figure S 4-1: Particle size distribution of Ag-NP measured by Dynamic Light Scattering (DLS) after 10 minutes, using a Malvern Zetasizer. The graph shows the result of three replicate analyses. Samples were sonicated for 30 seconds in a low power US bath.

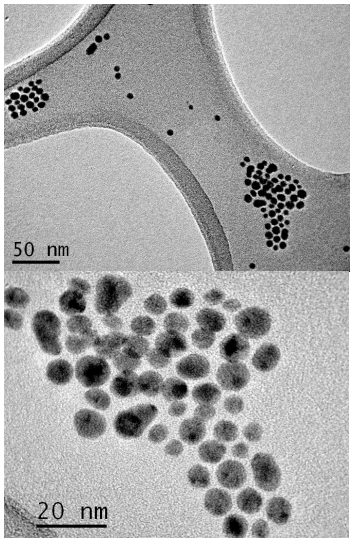

Figure S 4-2: Transmission electron microscopy (TEM) images of Ag NPs (3-8 nm) dispersed in pure water, using a JEOL 2010 analytical TEM. The solution was deposited on a holey carbon coated Cu TEM grid and dried at room temperature before examination.

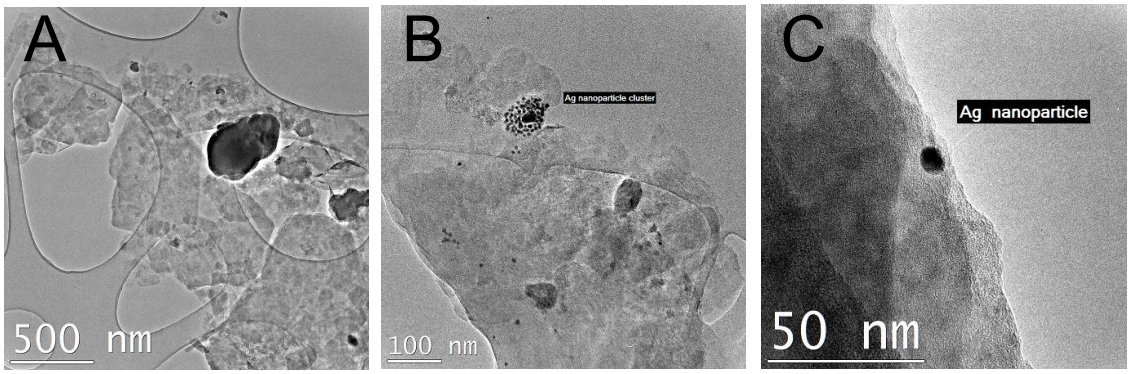

Figure S 4-3: TEM images of unspiked (A) and Ag NP spiked (nominal concentration 800 mg Ag/kg) (B, and C) Lufa 2.2 soil used for the toxicity tests with *Porcellionides pruinosus*. It was possible to detect Ag NP clusters (B), and single particles of 5-8 nm (C).

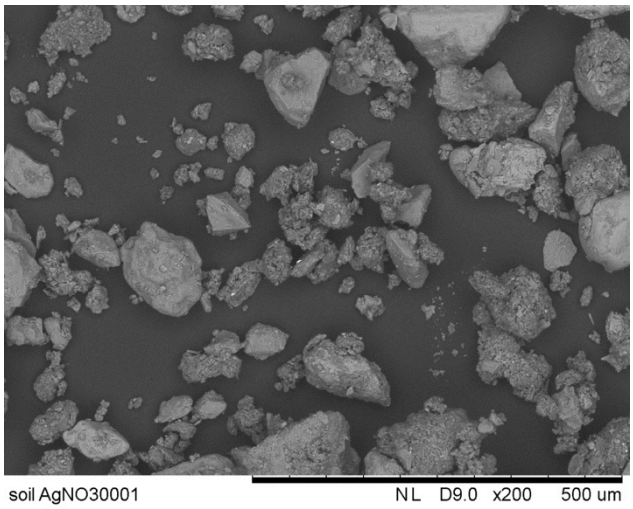

Figure S 4-4: SEM images of Lufa 2.2 soil spiked with $AgNO_3$ at 200 mg Ag/kg. Soil particles can be seen as the gray particles.

Table S 4-1: Ag concentrations (mg Ag/kg dry soil) measured in Lufa 2.2 soil spiked with Ag NPs and ionic Ag ($AgNO_3$) for the avoidance behavior test with the isopod *Porcellionides pruinosus*. Recovery (%) is presented in brackets.

Nominal concentration (mg Ag/kg dry soil)	Ag NPs	$AgNO_3$
Control	2.1	2.1
1	3.1 (306)	3.2 (322)
5	4.6 (92)	8.5 (171)
10	11.2 (112)	18.7 (187)
50	36.1 (72)	55.3 (111)
100	104 (104)	95.7 (96)
500	414 (83)	-

Table S 4-2: Ag nominal and measured concentrations (mg Ag/kg dry soil) in Lufa 2.2 soil spiked with Ag NPs and ionic Ag ($AgNO_3$) for the feeding inhibition tests with the isopod *Porcellionides pruinosus*. Soil pH_{CaCl2} was measured in freshly spiked soil. Mean Bioaccumulation Factor (BAF±SD; n=3) in isopods was calculated as tissue Ag concentration divided by the measured Ag concentration in soil. Recovery (%) is presented in brackets.

	Nominal concentration (mg Ag/kg dry soil)	Measured concentration	Soil pH_{CaCl2}	BAF
Ag NPs	Control	4.5	5.52	
	50	48.0 (96)	5.54	2.7 ± 0.26
	100	81.6 (82)	5.52	1.7 ± 0.57
	200	153 (77)	5.57	1.2 ± 0.41
	400	361 (90)	5.53	0.84 ± 0.01
	800	445 (56)	5.81	1.2 ± 0.18
$AgNO_3$	Control	2.8	5.72	
	12.5	11.1 (89)	5.57	4.7 ± 0.83
	25	24.7 (99)	5.45	3.7 ± 0.98
	50	33.4 (67)	5.40	2.8 ± 0.95
	100	68.1 (68)	5.41	2.1 ± 0.39
	200	252 (126)	5.33	1.5 ± 0.23

Table S 4-3: Ag concentrations (mg Ag/kg dw food) measured in alder leaves used for the feeding inhibition test with *Porcellionides pruinosus*. Recovery (%) is presented in brackets.

Nominal concentration (mg Ag/kg)	AgNPs	$AgNO_3$
Control	0.23	0.23
30	29 (95)	22 (72)
100	114 (114)	62 (62)
300	218 (73)	279 (93)
1000	905 (90)	785 (78)
3000	1499 (50)	1159 (39)

CHAPTER 5: TOXICOKINETICS OF Ag IN THE TERRESTRIAL ISOPOD *Porcellionides pruinosus* EXPOSED TO Ag NPs AND $AgNO_3$ VIA SOIL AND FOOD

Paula S. Tourinho[1], Cornelis A.M. van Gestel[2], A. John Morgan[3], Peter Kille[3], Claus Svendsen[4], Kerstin Jurkschat[5], J. Fred W. Mosselmans[6], Amadeu M.V.M. Soares[1], Susana Loureiro[1]

[1]Department of Biology and the Centre for Environmental and Marine Studies, University of Aveiro, Aveiro, Portugal

[2]Department of Ecological Science, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

[3]Cardiff School of Biosciences, BIOSI 1, University of Cardiff, P.O. Box 915, Cardiff, CF10 3TL, UK

[4]Centre for Ecology and Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, Oxfordshire OX10 8BB, UK

[5]Department of Materials, Oxford University, Begbroke Science Park, Sandy Lane, Yarnton, Oxford, OX5 1PF, UK

[6]Diamond Light Source Ltd., Harwell Science and Innovation Campus, Didcot, UK

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5.1 Abstract

Silver nanoparticles (Ag NPs) have been used in numerous consumer products and may enter the soil through the land application of biosolids. However, little is known about the relationship between Ag NP exposure and their bioavailability for soil organisms. This study aims at comparing the uptake and elimination kinetics of Ag upon exposures to different Ag forms (NPs and ionic Ag (as $AgNO_3$)) in the isopod *Porcellionides pruinosus.* Isopods were exposed to contaminated Lufa 2.2 soil or alder leaves as food. Uptake and elimination rate constants for soil exposure did not significantly differ between Ag NPs and ionic Ag at 30 and 60 mg Ag/kg. For dietary exposure, differences in the uptake rate constants for Ag NPs and $AgNO_3$ were related to feeding activity and exposure concentrations, while no difference in the elimination rate constants was found. When comparing both routes, dietary exposure resulted in lower uptake rate constants but elimination rate constants did not differ. A fast Ag uptake was observed from both routes and most of the Ag taken up seemed not to be eliminated. Our results show that isopods have an extremely high Ag accumulation capacity, suggesting the presence of an efficient Ag storage compartment.

Keywords: Ag nanoparticles, bioaccumulation, exposure route, isopods

5.2 Introduction

Silver nanoparticles (Ag NPs) have been used extensively in industry over the last decades, especially due to their bactericidal properties (Durán et al., 2007). They are embedded in consumer products such as textiles, cosmetics, food packaging, and in materials and devices for medical purposes (Hendren et al., 2011).

The application of Ag NPs may result in the release of Ag from the consumer products, ending up in the aquatic environment, including the wastewater (Benn et al., 2010; Kim et al., 2010). In wastewater treatment plants, Ag nanoparticles may be present in effluents and sewage sludge (Kaegi et al., 2011). In many countries, through the use of treated sewage sludge or biosolids as an agricultural amendment (Keller et al., 2013), Ag nanoparticles and other Ag species may be applied directly to soils. The land application of biosolids is considered the main route of Ag NPs to the soil environment (Gottschalk et al., 2009), where soil organisms live.

To relate exposure of chemicals to bioavailability, uptake and elimination kinetic studies are useful. In a typical study, organisms are exposed to non-toxic concentrations during an uptake phase, followed by a depuration or elimination phase in clean medium. Body concentration of the chemical is measured in the test organisms at several points in time and a kinetic model is fit to the data. Uptake and elimination kinetic parameters, bioaccumulation factor (BAF) and half-life of chemicals can be obtained from these kinetic models. In this way, not only can kinetic studies provide valuable information for risk assessment (Nahmani et al., 2007), it can also be very useful for regulatory purposes (Gobas and Morrison, 2000).

The bioaccumulation of Ag NPs and ionic Ag (from $AgNO_3$) has been evaluated in the earthworm *Eisenia fetida* after 28 days of exposure (Schlich et al., 2013; Shoults-Wilson et al., 2011). Schlich et al. (2013) showed slightly higher bioaccumulation factors (BAF) in earthworms exposed to Ag NPs, although the free Ag ion concentration in the soil was comparable for both Ag forms. Shoults-Wilson et al. (2011) found a lower BAF for Ag NP exposure, however, Ag bioaccumulation was not related only to Ag ions released from the nanoparticles. Up-to-date, only one study on the kinetics of Ag NPs in the springtail *Folsomia*

candida is available in the literature (Waalewijn-Kool et al., 2014). Ag NPs bioaccumulation in the springtails was found to be lower than its ionic counterpart (as $AgNO_3$), but dissolved Ag concentration in the pore water could not explain total Ag uptake from Ag NPs (Waalewijn-Kool et al., 2014).

As the bioaccumulation process may differ between organisms (Ardestani et al., 2014), assessing Ag NP bioaccumulation in different organisms is essential to get a complete picture of their potential threats to the environment. For this purpose, isopods are suitable indicators of metal bioavailability (Dallinger et al., 1992; Hopkin et al., 1986; Loureiro et al., 2002; Udovic et al., 2009). Kinetic studies with isopods are considered a better indicator of bioavailability than body burden measurements, since the flux of the contaminants into the organisms is more important in determining toxicity than total body concentration (van Straalen et al., 2005).

The aim of the present study is to evaluate the uptake and elimination kinetics of Ag in the terrestrial isopod *Porcellionides pruinosus*, considering two forms of Ag: ionic (with $AgNO_3$) and nanoparticulated (Ag NPs). As different routes of exposure are essential to fully evaluate the bioavailability of contaminants in the environment (Ardestani et al., 2014; Loureiro et al., 2002; Sousa et al., 2000; Vijver et al., 2004; Vink et al., 1995), kinetic studies were conducted using soil and dietary exposures for comparison.

5.3 Methodology

5.3.1 Test species and test chemicals

Specimen of the isopod *Porcellionides pruinosus* were collected in horse manure from an uncontaminated area in Coimbra (Portugal). The animals were kept in the laboratory at 20±2ºC and a 16/8h photoperiod for at least one month before the experiments. Adult males and non-gravid females (15-25 mg) in which no moulting process could be observed were used.

Ag NPs and ionic Ag (as $AgNO_3$) were used to spike soil and food. Ag NPs (AMEPOX) were supplied dispersed in water at 1 g/L. They had a diameter size of 3-8 nm (Figure S 5-1), with an alkane coating. For comparison to the ionic form, the soluble salt $AgNO_3$ (Sigma-Aldrich, 99% purity) was chosen.

5.3.2 Experimental setup – soil exposure

Lufa 2.2 soil (LUFA-Speyer 2.2, Sp 2121, LUFA Speyer, Speyer, Germany) was used in the kinetic tests and characterized as sandy loam with an organic carbon content of 2.3 ± 0.2%, pH_{CaCl2} of 5.6 ± 0.4, cation exchange capacity (CEC) of 10.0 meq/100 g and water-holding capacity (WHC) of 46.5%. Lufa 2.2 soil was spiked with Ag NPs or $AgNO_3$ as aqueous solution to reach two nominal concentrations of 30 and 60 mg Ag/kg dry soil. Additional water was added to reach 45% of the maximum WHC. Spiked soil was left one day for equilibration before the exposures started. Soil pH_{CaCl2} was measured at the beginning of the experiment by shaking 5 g of soil with 25 ml 0.01 M $CaCl_2$ solution for 2 hours and measuring pH in the solution after settling of the soil particles.

The kinetic experiment consisted of two phases. During the uptake phase the animals were exposed to Ag-spiked soil for 21 days. Then, the animals were transferred to unspiked soil for a 21-day elimination phase. The animals were kept individually in plastic boxes (⌀ 65 mm) and fed alder leaves *ad libitum*. At time points of 1, 2, 4, 7, 10, 14, and 21 days of each phase, three isopods were sacrificed for Ag body concentration measurements. Isopods and soil were stored at -20ºC until Ag analysis.

5.3.3 Experimental setup – dietary exposure

Alder (*Alnus glutinosa*) leaves were cut into disks (⌀ 10 mm) and separated in groups by dry weight (d.w.). Two groups, containing ~40 leaf disks each, were soaked into 400 ml of Ag NPs dispersed in water at 10 or 20 mg Ag/L and shaken (150 rpm) for 4 days. The same procedure was conducted with $AgNO_3$ solutions to obtain ionic Ag dosed food. The leaves were left to dry at room temperature for one day before being offered as food. Isopods were placed individually in chambers containing a net and a plaster bottom (for details, see Loureiro et al. (2008)). For the uptake phase, Ag dosed alder leaves were offered *ad libitum* to the isopods for 21 days. After that, the Ag-dosed leaves were replaced by undosed leaves during the 21-day elimination phase. After 1, 2, 4, 7, 10, 14, and 21 days of uptake and elimination, food was removed from the chambers and the

isopods were left in the chamber to empty their gut for one day. Then, animals were weighted and stored at -20ºC until total Ag analysis. The remaining food and faeces were dried at 45ºC for at least two days and their weight was recorded.

5.3.4 Total Ag analysis

Total Ag concentrations in isopods, soil and food were measured by Atomic Absorption Spectrometry (AAS). Soil (~130 mg) and leaf (~30 mg) samples were dried at 50ºC and then digested for 7 h in a mixture of concentrated HCl (J.T. Baker. purity 37%) and HNO_3 (J.T. Baker. purity 70%) (4:1. v/v) for 7 hours in closed Teflon containers, in an oven (CEM MDS 81-D) at 140ºC. After digestion, the samples were taken up in 10 mL of demineralized water and analysed for Ag by flame atomic absorption spectrometry (AAS; Perkin-Elmer AAnalyst 100). Soil samples, procedural blanks and reference material were analysed in duplicates, while leaf samples were analysed in one replicate due to the lack of material. Limit of detection (LOD) was 0.003 mg Ag/L, calculated as 3 times the standard deviation of the analytical standard blanks (n=20). Certified reference material (ISE sample 989 of River Clay from Wageningen, The Netherlands) was used to ensure the accuracy of the analytical procedure for soil samples. Recovery of Ag from the reference material was 100% (2.8 mg Ag/kg).

Isopods were freeze-dried, individually weighed and digested with a mixture of concentrated HNO_3:$HClO_4$ (7:1. v/v; J.T. Baker. ultrapure). The samples were evaporated to dryness and the residues were taken up in 1 mL 1M HCl. Ag content was determined by graphite furnace AAS (Perkin-Elmer 5100 PC). Procedural blanks were analysed in triplicates. LOD was found to be 0.039 ug Ag/L (n=20).

5.3.5 Toxicokinetic models

Two kinetic models were tested to describe the uptake and elimination rates in isopods, named here as models 1 and 2. Model 1 is a classic first-order one-compartment model, in which the animals are considered as one unique compartment. Model 2 is also a first-order one-compartment model adapted from Vijver et al. (2006). In this model, an inert fraction is considered in the organism in

which metals are stored and not eliminated during the elimination phase (Vijver et al., 2006).

In both models, uptake and elimination equations were fitted simultaneously. As metals naturally occur in the environment, the use of a background body concentration (C_0) is recommended (Skip et al. 2014). Therefore, C_0 was fixed by calculating the mean measured Ag body concentration at t=0.

For the uptake phase, the following equation was used in both models 1 and 2:

$$Q_{(t)} = C_0 + \frac{k1}{k2} \times C_{exp} \times (1 - e^{-k2 \times t}) \quad (1)$$

where Q(t) = Ag internal concentration at t days (μg Ag/g_{animal}); C_0 = background internal concentration (μg Ag/g_{animal}); k1 = uptake rate constant ($g_{soil/food}$ / g_{animal} / day); k2 = elimination rate constant (day^{-1}); C_{exp} = Ag exposure concentration (mg Ag/$kg_{soil/food}$); and t = time (days).

For the elimination phase, two different equations were used in model 1 (Eq.2) and model 2 (Eq.3 – adapted from Vijver et al. (2006)), as follows:

$$Q_{(t)} = C_0 + \frac{k1}{k2} \times C_{exp} \times \left(e^{-k2 \times (t - t_c)} - e^{-k2 \times t}\right) \quad (2)$$

$$Q_{(t)} = C_0 + \frac{k1}{k2} \times C_{exp} \times \left[Fi + (1 - Fi) \times \left(e^{-k2 \times (t - t_c)}\right)\right] \quad (3)$$

where t_c = time the animals are transferred to clean medium (days) and F_i = inert fraction (ranging from 0 to 1).

For Ag NP exposure at 30 mg Ag/kg, it was necessary to constraint the parameter Fi to be <1.00, since Fi ranges from 0 to 1 (Vijver et al. 2006).

5.3.6 Statistical analysis

Uptake and elimination equations were fitted to the data and kinetics parameters were estimated using non-linear regression in SPSS (version 20). An F test was

run to determine which of the two non-linear models with different number of parameters gave the best fit (Motulsky and Ransnas, 1987). Differences in k1 and k2 were tested by a Generalized Likelihood Ratio test and overlap of 95% confidence intervals, respectively. Half-life for elimination of Ag from the isopods after exposure to both test compounds was calculated as ln(2)/k2, and Ag bioaccumulation factor (BAF) as k1/k2.

In the dietary exposure, feeding activity expressed as food consumption ratio, assimilation ratio and assimilation efficiency was calculated in accordance to Loureiro et al. (2006). Feeding parameters were correlated to Ag body concentration in the isopods measured during the uptake phase using non-parametric Spearman correlation analysis in SPSS (version 20).

5.4 Results

5.4.1 Soil properties and Ag measurements

Soil pH_{CaCl2} in Ag spiked soils ranged from 5.47 to 5.50 (Table S 5-1) and did not change in comparison to unspiked Lufa 2.2 soil (pH 5.49). Good recovery was obtained for Ag measurements in all soil samples, ranging from 81 to 124% (Table S 5-1). Nominal concentrations of 30 and 60 mg Ag/kg resulted in measured concentrations of 37 and 48 mg Ag/kg for Ag NPs, and 27 and 70 mg Ag/kg for ionic Ag, respectively.

Ag concentration in alder leaves spiked with Ag NPs was found to be lower in comparison to leaves spiked with $AgNO_3$. For Ag NPs, concentrations were 534 and 832 mg Ag/kg_{food}, while for ionic Ag concentrations were 4499 and 4717 mg Ag/kg_{food}.

All toxicokinetic calculations in this paper are based on measured concentrations in soil and food.

The background of Ag concentration found in isopods (T0) was 2.30±1.32 µg Ag/g (mean±SD, n=3).

5.4.2 Uptake and elimination kinetics – soil exposure

No isopod mortality and weight change was observed during the 42-day experimental period (data not shown). After 21 days of uptake, mean Ag body concentration reached 90 and 136 µg Ag/g in isopods exposed to Ag NPs at 37 and 48 mg Ag/kg in soil, respectively. For ionic Ag, mean body concentration was 84 and 164 µg Ag/g at 27 and 70 mg Ag/kg, respectively. According to model 1, steady state body concentration was not reached in isopods exposed for 21 days to both Ag forms in soil during the uptake phase, although model 2 does suggest that steady state was reached (Figure 5.1).

Ag kinetics parameters obtained by fitting model 1 (one-compartment model) and model 2 (one-compartment model with an inert fraction) are provided in Table 5-1. Best fit was obtained by model 2 for Ag NPs at 37 ($F_{1,39}$=19.99, $p<0.05$) and 48 mg Ag/kg ($F_{1,39}$=20.37, $p<0.05$), and for $AgNO_3$ at 27 ($F_{1,40}$=6.99, $p<0.05$) and 70 mg Ag/kg ($F_{1,38}$=13.87, $p<0.05$). For that reason, model 2 was chosen to describe the uptake and elimination kinetics of Ag in soil exposures. The fit of the models to Ag body concentration over time can be found in Figure 5.1 (model 2) and Figure S 5-2 (model 1).

Using model 2, the uptake rate constant k1 was 0.79 and 0.57 g_{soil} / g_{animal} / day for Ag NPs at 37 and 48 mg Ag/kg, and 0.46 and 0.48 g_{soil} / g_{animal} / day for ionic Ag at 27 and 70 mg Ag/kg, respectively. Values of k1 did not differ between the two exposure concentrations for Ag NPs ($X^2_{(1)}$= 0.75; n.s.) and ionic Ag ($X^2_{(1)}$< 0.01; n.s.). Moreover, no significant difference in k1 was found between both Ag forms at the two lower exposure concentrations (37 and 27 mg Ag/kg for AgNPs and $AgNO_3$, respectively) ($X^2_{(1)}$= 1.30; n.s.) and the two higher concentrations (48 and 70 mg Ag/kg for AgNPs and $AgNO_3$, respectively) ($X^2_{(1)}$= 0.45; n.s.).

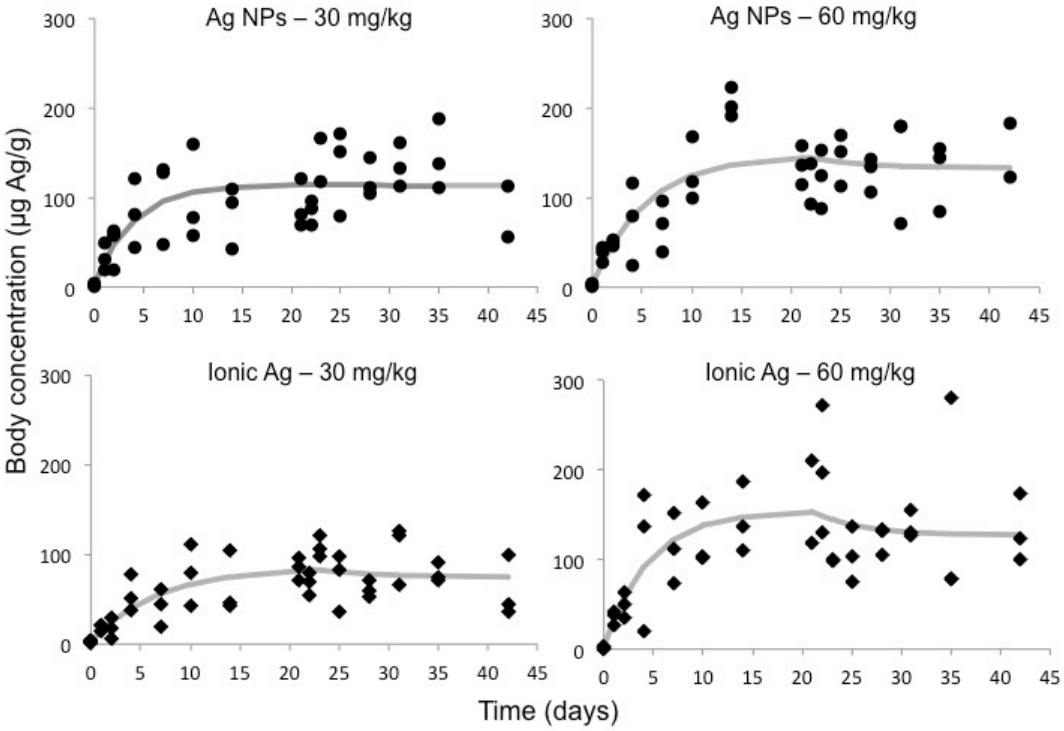

Figure 5.1: Uptake and elimination kinetics of Ag from Ag NPs (circles) and ionic Ag as $AgNO_3$ (diamonds) in the isopod *Porcellionides pruinosus* exposed to nominal concentrations of 30 and 60 mg Ag/kg in Lufa 2.2 soil. Uptake and elimination phases lasted for 21 days each. Lines represent the modelled Ag body concentration, using model 2 (equations 1 and 3).

Elimination rate constant k2 was 0.26 and 0.19 day^{-1} for Ag NPs at 37 and 48 mg Ag/kg, and 0.15 and 0.22 day^{-1} for ionic Ag at 27 and 70 mg Ag/kg, respectively. No significant difference in k2 values was found between concentrations and Ag forms, based on overlap of the 95% of confidence intervals. The inert fraction (Fi) values were 0.99 and 0.90 for Ag NPs at 37 and 48 mg Ag/kg, respectively. For ionic Ag, Fi values were 0.87 and 0.82 at 27 and 70 mg Ag/kg, respectively.

Although it is suggested by model 1 that steady state Ag body concentrations were not reached, bioaccumulation factors (BAF) were calculated. BAF values were 3.0 for Ag NPs at both exposure concentrations, while BAF was 3.1 and 2.2 for ionic Ag at 27 and 70 mg Ag/kg, respectively.

5.4.3 Uptake and elimination kinetics – dietary exposure

Some mortality was observed in isopods during the food-exposure experiment, ranging from 11 to 16%. Possibly the moisture maintenance provided by the

plaster bottom of the experimental chambers was not efficient enough and caused this mortality. Still, the fresh weight of surviving animals did not significantly change after the 42 days of experiment, indicating good health of the test animals (data not shown).

Mean Ag body concentration ranged from 370 to 414 µg Ag/g in isopods exposed to Ag NPs and from 435 to 658 µg Ag/g in isopods exposed to ionic Ag after 21 days of uptake. Model 2 failed to fit the data, so model 1 was used to describe Ag kinetics upon dietary exposure (Figure 5.2).

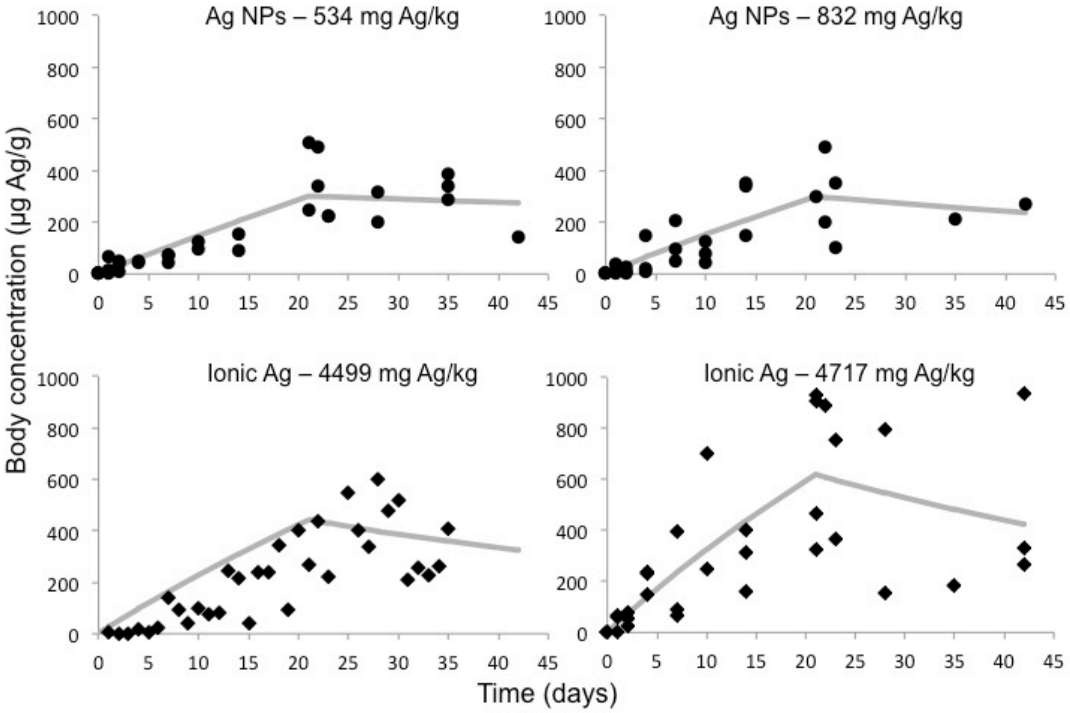

Figure 5.2: Uptake and elimination kinetics of Ag NPs (circles) and ionic Ag as $AgNO_3$ (diamonds) in the isopod *Porcellionides pruinosus* exposed to Ag spiked alder leaves as food. Uptake and elimination phases lasted for 21 days each. Lines represent the modelled Ag body concentration, using model 1 (equations 1 and 2).

Uptake rate constant k1 was 0.028 and 0.019 g_{food} / g_{animal} / day for Ag NPs at 534 and 832 mg Ag/kg_{food}, respectively. Lower k1 values were found for ionic Ag of 0.005 and 0.008 g_{food} / g_{animal} / day at 4499 and 4717 mg Ag/kg_{food}, respectively (Table 5.2). k1 did not significantly differ between the two exposure concentrations for Ag NPs ($X^2_{(1)}$=2.74; n.s.) and ionic Ag ($X^2_{(1)}$=2.91; n.s.).

Table 5.1: Uptake and elimination kinetic parameters for Ag nanoparticles (NPs) and ionic Ag in isopods (*Porcellionides pruinosus*) exposed to Lufa 2.2 soil at nominal concentrations of 30 and 60 mg Ag/kg. 95% confidence intervals are given in between brackets

Model	Ag form	Nominal concentration (mg/kg)	Measured concentration (mg/kg)	k1 (g_{soil}/g_{animal}/ day)	k2 (day^{-1})	Fi	BAF	half-life (days)
model 1	Ag NPs	30	37	0.19 (0.12-0.25)	0.01 (0.00–0.33)		16.2	59.6
		60	48	0.21 (0.15-0.27)	0.03 (0.00-0.04)		8.4	27.2
	Ionic Ag	30	27	0.21 (0.13-0.29)	0.02 (0.00-0.04)		9.7	31.7
		60	70	0.17 (0.11-0.22)	0.03 (0.00-0.05)		5.3	22.2
model 2	Ag NPs	30	37	0.79 (0.028-1.30)	0.26 (0.06-0.46)	0.99	3.0	
		60	48	0.57 (0.12-1.01)	0.19 (0.05-0.32)	0.91	3.0	
	Ionic Ag	30	27	0.46 (0.07-0.85)	0.15 (0.03-0.27)	0.87	3.1	
		60	70	0.48 (0.19-0.78)	0.22 (0.06-0.39)	0.82	2.2	

k1-uptake rate constant; k2-elimination rate constant; Fi-inert fraction; BAF-bioaccumulation factor; model 1- Equations 1 and 2; model 2 – Equations 1 and 3

Elimination rate constant (k2) was 0.004 and 0.011 day^{-1} for Ag NPs at 534 and 832 mg/kg_{food}, respectively. For ionic Ag, k2 was 0.015 and 0.018 day^{-1} at 4499 and 4717 mg/kg_{food}, respectively. The 95% confidence intervals for the k2 values for the different treatments overlapped (Table 5-2), suggesting no difference between treatments. However, k2 values were slightly lower for Ag NPs, resulting in higher BAF and half-life values compared to the ionic Ag exposures.

Table 5-2: Uptake and elimination kinetic parameters for Ag nanoparticles (NPs) and ionic Ag (as $AgNO_3$) in isopods (*Porcellionides pruinosus*) exposed to Ag-spiked alder leaves. Parameters were calculated using a one-compartment model (Equations 1 and 2). 95% confidence intervals are in brackets

	Concentration ($mg\ Ag/kg_{food}$)	k1 (g_{food} / g_{animal} / day)	k2 (day^{-1})	BAF	Half-life (days)
Ag NPs	534	0.028 (0.016 - 0.40)	0.004 (0.00– 0.025)	6.5	161
	832	0.019 (0.009 - 0.29)	0.011 (0.00 - 0.039)	1.7	61.9
Ionic Ag	4499	0.005 (0.004 - 0.007)	0.015 (0.001 - 0.028)	0.37	47.0
	4717	0.008 (0.00 - 0.012)	0.018 (0.00 - 0.044)	0.41	38.2

k1-uptake rate constant; k2-elimination rate constant; a – assimilation rate as Cexp * k1; BAF-bioaccumulation factor

Food consumption ratio reached up to 0.21 and 0.28 mg food/mg isopod after 21-d of exposure to Ag NPs at 534 and 832 $mg\ Ag/kg_{food}$, respectively (Figure S 5-3). For $AgNO_3$, maximum food consumption ratio was 0.09 and 0.11 mg food/mg isopod after 21 days of exposure at 4499 and 4717 $mg\ Ag/kg_{food}$, respectively. Higher assimilation ratios were also observed in the Ag NP treatments, while assimilation efficiency was comparable between Ag NP and $AgNO_3$ treatments (Figure S 5-2). Egestion ratio was also found to be higher in isopods exposed to Ag NPs (data not shown) and was strongly influenced by consumption ratio (i.e., faecal production increased with increasing food consumption).

Ag NP and ionic Ag treatments showed similar patterns when relating feeding parameters with Ag body concentrations (Table 5.3; Figure S 5-3). A positive significant relationship was found between food consumption ratio and Ag body concentrations for all treatments (Spearman test, $p<0.05$). No significant relationship was found between assimilation ratio and Ag body concentrations (Spearman test, $p>0.05$), and a weak negative significant relationship was found between assimilation efficiency and Ag body concentrations in all treatments (Spearman test, $p<0.05$).

Table 5-3: Spearman correlation coefficients (r^2) for the relation between feeding parameters (consumption ratio, assimilation ratio, assimilation efficiency) and Ag body concentrations in isopods (*Porcellionides pruinosus*) exposed to Ag NPs and ionic Ag contaminated food. Food concentration was 534 and 834 mg Ag/kg dry food for Ag NPs and 4499 and 4717 mg Ag/kg dry food for ionic Ag. Asterisks indicate significant correlation ($p<0.05$).

Ag form	mg Ag/kg	Feeding activity		
		Consumption ratio	Assimilation ratio	Assimilation efficiency
Ag NPs	534	0.83*	0.46	-0.66*
	832	0.79*	0.32	-0.60*
Ionic Ag	4499	0.84*	0.20	-0.67*
	4717	0.85*	0.16	-0.57*

5.5 Discussion

5.5.1 Soil exposure

Upon soil exposure, Ag concentrations in the isopods increased with time for both Ag NPs and ionic Ag. This probably is due to the fact that the animals were hardly able to eliminate Ag, as little or no decrease in Ag body concentration was observed in the elimination phase (Figure 5.1).

Uptake and elimination rate constants showed no significant differences between the two Ag forms. This could be a result of the route of exposure in hard-bodied organisms like isopods, which may be less exposed to the soluble fractions

present in pore water (van Gestel and van Straalen, 1994). Uncoated Ag NPs and ionic Ag were found to have different time-dependent behaviour in soils (Coutris et al., 2012). The Ag water-extractable and ion-exchangeable fractions were found to increase with time in soils spiked with Ag NPs, while they decreased in soils spiked with $AgNO_3$ (Coutris et al., 2012). Nevertheless, low porewater Ag concentrations were observed in Lufa 2.2 soil spiked with Ag NPs and $AgNO_3$ (Waalenwijn-Kool et al. 2014).

Upon soil exposure, Ag kinetics from both Ag NPs and $AgNO_3$ were best described by a one-compartment-model with an inert fraction (model 2). The inert fraction represents the metal storage fraction from which no metal elimination occurs during the elimination phase (Vijver et al., 2006). In isopods, metal storage takes place in S-cells and B-cells of the hepatopancreas (Hames and Hopkin, 1989). These cells differ in their functions, with the B-cells being responsible for metal absorption and elimination, while the S-cells are responsible for only absorption (Donker et al., 1996). This means that metals accumulated in the S-cells may never be eliminated and are stored for a whole lifetime (Hames and Hopkin, 1991). Accumulation in these cells may also be metal-specific. For instance, Cd is accumulated in S-cells, while Zn is accumulated in both B and S-cells (Hopkin, 1990a). In the present study, the slow elimination of Ag suggested that most of Ag accumulation is likely to take place in the S-cells, from which no elimination occurs.

The high inert fraction (Fi) observed in the isopods confirms this hypothesis. Fi values were found to be 0.99 and 0.90 for Ag NPs and 0.87 and 0.82 for ionic Ag upon exposure to nominal concentrations of 30 and 60 mg Ag/kg dry soil, respectively. These values are well in agreement with other studies that have shown that up to 90% of total metal accumulated in isopods is detected in the hepatopancreas (Hopkin, 1990b). The isopod *Porcellio laevis* exposed to Cd-spiked food had >90% of the total Cd in the hepatopancreas (Odendaal and Reinecke, 1999; Odendaal and Reinecke, 2004). And when exposed to Zn-spiked food, the isopod *Porcellio scaber* had up to 70% (Donker et al., 1996) and 99% (Odendaal and Reinecke, 2004) of total Zn accumulated in the hepatopancreas. van Straalen et al. (2005) showed that the amount of Zn in the hepatopancreas is

dependent on exposure concentration, with between 40-60% and 80-90% of Zn in the hepatopancreas at exposure concentrations of 200 and 1,500 mg/kg dry food, respectively. Vijver et al. (2006) also found relatively low Fi values of 0.43 and 0.55 for Cd and Zn in *P. scaber* upon soil exposure, respectively.

The elimination rate constants for Ag found in this study were independent neither of the exposure route nor of Ag exposure levels or Ag form. Interestingly, the Ag elimination rate constants were also in close agreement with the k2 values for Cd and Zn in the isopod *P. scaber* reported by Vijver et al. (2006). The k2 values for *P. scaber* were 0.19 and 0.18 day-1 for Cd and Zn exposures, respectively (Vijver et al., 2006), while k2 values for Ag in *P. pruinosus* varied from 0.15 to 0.26 day-1 for both the nanoparticulate and ionic Ag forms.

Waalewijn-Kool et al. (2014) performed a toxicokinetic study on Ag in the springtail *Folsomia candida*. Springtails were exposed to similar conditions as in the present study, with Lufa 2.2 soil spiked with Ag NPs (AMEPOX, 3-8 nm) and $AgNO_3$ as ionic Ag. The uptake rate constant was about 0.10 gsoil / ganimal / day for Ag NPs at 140 mg Ag/kg dry soil, and 0.17 and 0.09 gsoil / ganimal / day for $AgNO_3$ at 30 and 60 mg Ag/kg dry soil (Waalewijn-Kool et al., 2014). The elimination rate constant was 0.09 day-1 for Ag NPs and varied between 0.01-0.03 day-1 for $AgNO_3$ (Waalewijn-Kool et al., 2014). Lower Ag elimination from $AgNO_3$ was observed in the springtails, resulting in higher BAF and half-life values in comparison to Ag NPs (Waalewijn-Kool et al., 2014). The same was not observed in our study, where k2 values did not differ between Ag NPs and $AgNO_3$. It may suggest that isopods and springtails have different bioaccumulation kinetics for Ag NPs and $AgNO_3$. While isopods showed comparable accumulation and elimination capacity between Ag NPs and $AgNO_3$, springtails showed lower elimination for ionic Ag than for Ag NPs. In fact, in our study, a slightly higher inert fraction of Ag was found in isopods exposed to Ag NPs (0.90-0.99) when compared to isopods exposed to $AgNO_3$ (0.82-0.87). This might suggest a slightly different mechanism of accumulating both Ag forms.

5.5.2 Dietary exposure

Silver uptake and elimination kinetics for Ag NPs and $AgNO_3$ from contaminated food was described by a one-compartment model (model 1). Due to the spiking

procedure (i.e. leaves soaked in Ag solution), final Ag concentrations were ~8.5 and 5.5 higher in food spiked with $AgNO_3$ than with Ag NPs, when soaked into solutions containing 10 and 20 mg Ag/L, respectively.

Higher uptake rate constants for Ag were found in isopods exposed to Ag NPs. However, these differences could be explained by the food consumption ratio. Isopods can avoid highly contaminated food by decreasing food consumption (Drobne and Hopkin, 1995; Loureiro et al., 2006; Zidar et al., 2012). Thus, the higher Ag concentration in animals offered $AgNO_3$-spiked food could be related to their lower food consumption (see Figure S 5-3).

The food assimilation ratio (mg assimilated food/ mg isopod) was not related with Ag body concentration. During food digestion, fluids and fine particles are separated from coarse particles in the primary and secondary filters in the proventriculus, going to the hepatopancreas afterwards. The coarse particles are voided and eliminated as faecal pellets after the digestive process (Hames and Hopkin, 1989). It is known that metals taken up with food may enter the body and reach the hepatopancreas without reaching other parts of the body (Vijver et al., 2006). This may explain the lack of a relationship between food assimilation and Ag assimilation (assessed as body concentration) in this study, considering that food and metals will show different assimilation pathways during the digestive processes.

Assimilation efficiency (percentage of assimilated food from ingested food) had a significant negative relationship with Ag body concentration for all treatments (Table 5-3). Higher assimilation efficiency indicates that the animal improved the nutritional gain from the ingested food. In this way, they avoided Ag uptake by consuming less contaminated food, and being less exposed to Ag.

No difference in the elimination rate constant was found between Ag NPs and $AgNO_3$. This is not surprising since k2 will be rather dependent on the organism (Crommentuijn et al., 1994; Díez-Ortiz et al., 2010), while k1 will depend on the exposure conditions, like the available metal concentration and the medium characteristics (Crommentuijn et al., 1994).

Usually, low elimination rate constants for metals in isopods exposed via food are found. Elimination rate constant for Cd was found to be zero in the isopods *P.*

scaber and *Oniscus asellus* (Crommentuijn et al., 1994), and low elimination capacity of Cd and Zn was observed in the isopod *P. scaber* (Vijver et al., 2006). Also for Ag in the isopod *P. pruinosus* used in our study, elimination rates were low. This may result in a continuous increase of Ag accumulation in the isopods over time. The metal accumulation strategy in isopods is very efficient, nevertheless when storage limit is reached, it is very likely that toxicity will take place (Hopkin, 1990a; van Straalen et al., 2005).

5.5.3 Comparison between soil and dietary exposures

Oral uptake of contaminated soil and food is the main route of exposure in isopods (Koster et al., 2005; van Gestel and van Straalen, 1994; Vijver et al., 2006). Comparing the results obtained from model 1, uptake rate constants for Ag NPs and ionic Ag were lower upon dietary exposures than for soil exposures. Ag dietary uptake was controlled not only by exposure concentration, but also by the feeding activity (i.e., food consumption). It suggests that the avoidance of contaminated food, a typical behaviour found in isopods, may have a great influence on Ag uptake. In soil, exposure concentration and ingestion of contaminated soil was probably the main factor affecting Ag uptake. However, it is not possible to determine whether the isopods were capable or not of avoiding the ingestion of contaminated soil. Not much is known about the reason why isopods ingest soil particles, but it seems to be important for the digestive process in these organisms (Zimmer, 2002).

After ingestion of contaminated soil or food, the assimilation of pollutants in the body will be dependent on their bioavailability (i.e., desorption or dissolution from the medium inside the body). Organic matter content is an important factor that determines the bioavailability of metals when comparing soil and dietary exposures. Due to strong sorption of metals to organic matter, the high organic matter content in leaf material leads to greater sorption of metals when compared to soil (Sousa et al., 2000; Vink et al., 1995), especially of the loosely bound or free fractions of metal (Vijver et al., 2006). It may result in lower bioavailability of metals when exposed via food.

Elimination rate constants were found to be very low and comparable between both routes. It is suggested that elimination capacity in isopods is low and independent of the route of exposure. As uptake rate constants were higher upon soil exposure, higher BAF values for Ag were found in isopods exposed via soil.
Due to the low elimination capacity, Ag body concentrations remained almost constant during the elimination phase, and a significant part of the Ag seemed to be stored in an inert fraction. For soil exposures, it was possible to quantify this fraction using model 2, for food exposures model 2 could not be fitted to the data. Nevertheless, it is clear from the data that elimination is slow also upon food exposure, and this may again be attributed to storage of the Ag. The storage of non-essential metals (with no elimination) is a main detoxification strategy in soil organisms, including isopods (Vijver et al., 2004). Our results suggest that Ag from both the Ag NPs and $AgNO_3$ was stored by the isopods rather than being eliminated. Interestingly, Ag storage and elimination from Ag NPs and ionic Ag were quite similar, suggesting that Ag NPs were also taken up as ionic Ag. In agreement with our finding, assimilation of dissolved Ag from ingested NPs was observed in the isopod *P. scaber* exposed via food (Pipan-Tkalec et al., 2011).

5.6 Conclusions

High accumulation capacity of Ag from Ag NPs was observed in isopods exposed via soil and food. Our results suggest that Ag is accumulated in a storage compartment (i.e., hepatopancreas), as shown by the very low elimination rate constants. The elimination of Ag was independent on both exposure route and Ag form, showing that the storage strategy in isopods is a prevailing factor in Ag bioaccumulation. Due to slow elimination, Ag may be accumulated until it reaches toxic levels, and may pose a threat to terrestrial isopods in case of long-term exposure. Furthermore, because of the high levels accumulated in isopods, Ag might be transferred to possible predators. Biomagnification of Ag in the terrestrial environment therefore cannot be ruled out and should be further investigated.

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5.8 Supplementary material

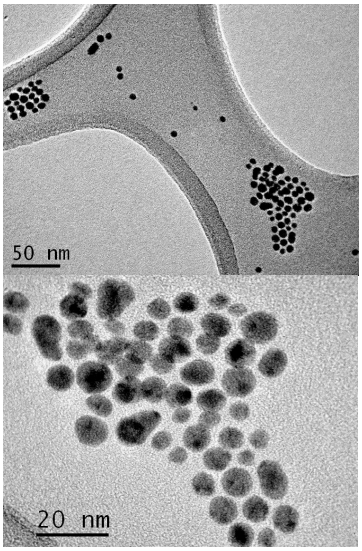

Figure S 5-1: TEM images of Ag NPs (AMEPOX, 3-8nm) in pure water.

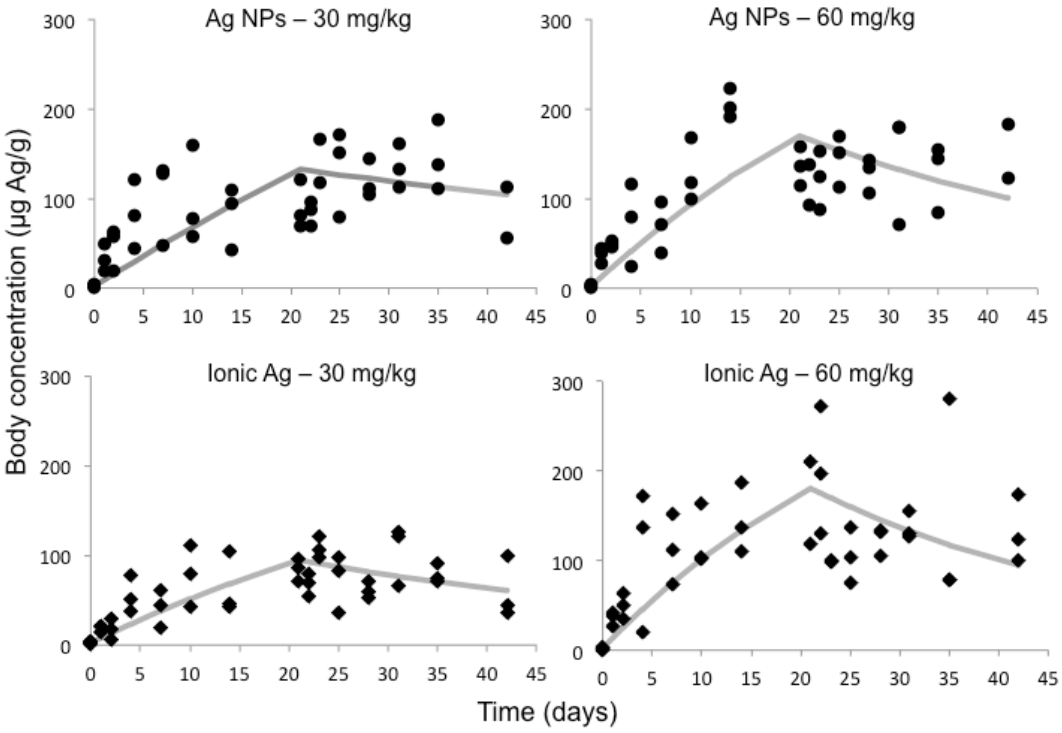

Figure S 5-2: Uptake and elimination kinetics of Ag from Ag NPs (circles) and ionic Ag as $AgNO_3$ (triangles) in the isopod *Porcellionides pruinosus* exposed to nominal concentrations of 30 and 60 mg Ag/kg in Lufa 2.2 soil. Uptake and elimination phases lasted for 21 days each. Lines represent the modelled Ag body concentration, using model 1 (equations 1 and 2).

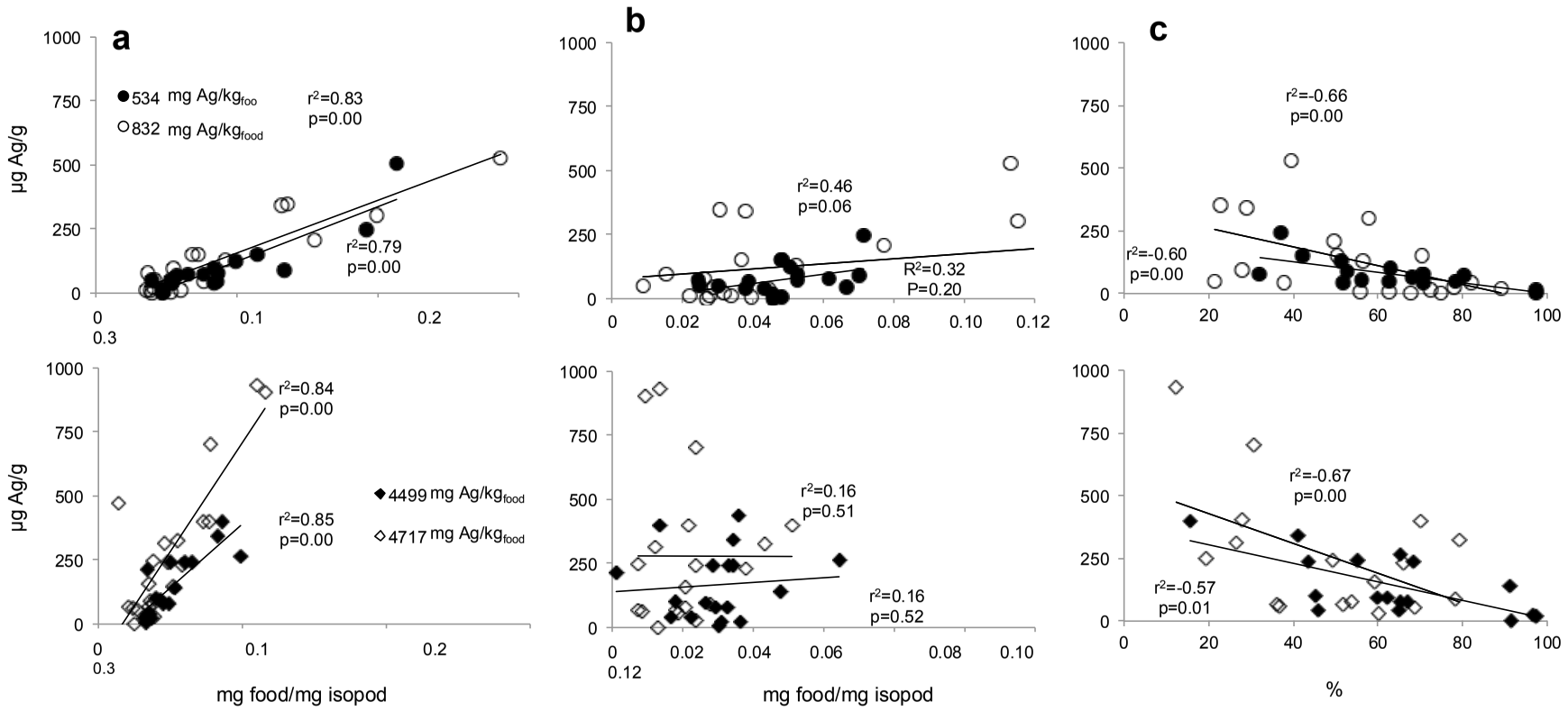

Figure S 5-3: Correlation coefficient (r^2) and p-value obtained by Spearman correlation analysis between Ag body concentration (µg Ag/g) and feeding activity in the isopod *Porcellionides pruinosus* exposed to food spiked with as Ag NPs (top) and $AgNO_3$ (bottom). Exposure concentrations in food were 534 and 832 mg Ag/ kg dry food (closed and open circles, respectively) for Ag NPs and 4499 and 4717 mg Ag/kg dry food (closed and open triangles, respectively). (A) Consumption ratio (mg food/mg isopod); (B) Assimilation ratio (mg food/mg isopod); (C) Assimilation efficiency (%).

Table S 5-1: Ag measured concentration (mean; n=2), recovery and pH_{CaCl2} in Lufa 2.2 soil spiked with Ag NPs and ionic Ag ($AgNO_3$) at 30 and 60 mg Ag/kg. Also given is the result of the Ag analysis in a certified reference material.

	Nominal concentration (mg Ag/kg)	Measured concentration (mg Ag/kg)	Recovery (%)	pH
Ag NPs	30	37	124	5.50
	60	48	81	5.49
$AgNO_3$	30	26	87	5.48
	60	70	116	5.47
Reference material*	3	3	100	-

* ISE sample 989 of River Clay from Wageningen (The Netherlands).

CHAPTER 6: CeO_2 NANOPARTICLES INDUCE NO CHANGES ON PHENANTHRENE TOXICITY TO THE ISOPOD *Porcellionides pruinosus*

Partly published in Tourinho et al. (2015). CeO_2 nanoparticles induce no changes on phenanthrene toxicity to the soil organisms *Porcellionides pruinosus* and *Folsomia candida.* Ecotoxicology and Environmental Safety, Vol. 113, pp. 201-206.

6.1 Abstract

Cerium oxide nanoparticles (CeO_2 NPs) are used as diesel fuel additives to catalyze oxidation. Phenanthrene is a major component of diesel exhaust particles and one of the most common pollutants in the environment. This study aimed at determining the effect of CeO_2 NPs on the toxicity of phenanthrene in Lufa 2.2 standard soil for the isopod *Porcellionides pruinosus*. Toxicity tests were performed in the presence of CeO_2 concentrations of 10, 100 or 1000 mg Ce/kg dry soil and compared with results in the absence of CeO_2 NPs. CeO_2 NPs had no adverse effects on isopod survival and growth. LC50s for the effect of phenanthrene ranged from 110 to 143 mg/kg dry soil, and EC50s from 17.6 to 31.6 mg/kg dry soil. From this study it may be concluded that CeO_2 NPs have a low toxicity and do not affect toxicity of phenanthrene to isopods.

Keywords: Cerium oxide nanoparticles; mixture toxicity; isopods

6.2 Introduction

Engineered nanoparticles (NPs) are defined as industrially produced materials in a size ranging from 1 to 100 nm at least in one of their dimensions, for 50% or more of the particles in the number size distribution (European Commission, 2011). New applications of NPs are constantly being developed, resulting in the introduction to the market of diverse products containing NPs (Roco et al., 2011). Within commercially available NPs, CeO_2 NPs have drawn attention due to their potential use as fuel additive in diesel (Cassee et al., 2011; Johnson and Park, 2012). CeO_2 NPs enhance the oxidation rate and decrease the emission of particulate matter during combustion (Jung et al., 2005). Diesel fuel additives show promising improvements for emission reductions (Park et al., 2008). Nevertheless, the potential release of CeO_2 NPs in combination with other emission products in the environment is still unknown. Also mixture toxicology of CeO_2 NPs and organic pollutants in soil has not been studied so far.

Predictions of CeO_2 NPs in soils showed an environmental concentration of 0.016 mg/kg in (20-meter) areas next to urban roads in the United Kingdom, after 7 years of deposition (Johnson and Park, 2012). CeO_2 NP concentrations are expected to increase up to 0.04 mg/kg after 12 years of deposition (Johnson and Park, 2012). Adverse effect concentrations, however, have shown to be several orders of magnitude above predicted concentrations in the environment (Batley et al., 2013; Johnson and Park, 2012; Park et al., 2008). Some ecotoxicological aquatic studies showed uptake of CeO_2 NPs in zebra fish (Johnston et al., 2010) and clustering of CeO_2 NPs around algal cells (Van Hoecke et al., 2011). Also, CeO_2¬ NPs may have genotoxic potential toward aquatic organisms like the freshwater crustacean *Daphnia magna* and larvae of the aquatic midge *Chironomus riparius* at 1 mg/L (Lee et al., 2009).

However, some studies have suggested that CeO_2 NPs could work as an antioxidant or free radical scavenger leading therefore to a low toxicity (Colon et al., 2010; Schubert et al., 2006; Xia et al., 2008). By comparing CeO_2 NPs and ZnO NPs, Xia et al. (2008) found different mechanisms of toxicity in cellular responses. While ZnO and TiO_2 generated reactive oxygen species (ROS) leading

to cell death, CeO_2 NPs showed a protective response by suppressing ROS production.

CeO_2 NPs are likely to co-exist with polycyclic aromatic hydrocarbons (PAHs), as both are released during the combustion process of diesel fuel. Nanoparticles have a proportionately very large surface area and this surface can have a high affinity for organic chemical combustion products such as PAHs (Moore, 2006). Few toxicity studies have assessed the effects of the combination of NPs and PAHs. Yang and Watts (2005) studied the toxicity of Al_2O_3 NPs when loaded with phenanthrene or unloaded to different plant species. Unloaded Al_2O_3 NPs were found to be more toxic, as lower root elongation was found (Yang and Watts, 2005). Hu et al. (2008) showed that fullerene C60 decreased the bioavailability of PAHs (phenanthrene, fluoranthene and chrysene) by decreasing the freely dissolved PAH concentration in water. However, in another study, phenanthrene sorbed to C60 was still available to the algae *Raphidocelis subcapitata* (previously known as *Pseudokirchneriella subcapitata)* and the crustacean *D. magna*, where higher toxicity of phenanthrene was observed in the presence of C60 (Baun et al., 2008).

Phenanthrene is a major component of diesel exhaust particles and therefore a common pollutant in the environment. Phenanthrene concentrations in soil vary between urban and unpolluted rural or forest areas, with urban soils presenting higher levels due to anthropogenic emissions. However, a large variation in phenanthrene concentrations in soils can be found. Phenanthrene concentrations were estimated at ~500 µg/kg in urban soils (Wild and Jones, 1995), ~700 µg/kg in street dust particles in Copenhagen (Johnsen et al., 2006), and only 43 µg/kg in urban areas of Tarragona County (Nadal et al., 2011). In unpolluted areas in the UK and Norway concentrations ranged between 42 to 54 µg/kg (Nam et al., 2008), while 7 µg/kg was measured in Tarragona County (Nadal et al., 2011).

No data on combined effects of CeO_2 NPs and phenanthrene is available in the literature for soil organisms. And for the isopod *Porcellionides pruinosus* no data on the toxicity of phenanthrene is available at all. This study therefore aimed at evaluating the influence of CeO_2 NPs on the toxicity of phenanthrene to the soil

organism, the isopod *P. pruinosus*. For that purpose, feeding inhibition test was performed with *P. pruinosus.*

Three scenarios are possible for the outcome of mixture toxicity tests with phenanthrene and CeO_2 NPs. In a first scenario, the toxicity of the mixture of phenanthrene and CeO_2 NPs is additive, with CeO_2 NPs showing almost no toxicity from the mixture of phenanthrene and CeO_2 NPs compared to phenanthrene alone. In a second scenario CeO_2 NPs act as a synergist and increase toxicity of phenanthrene. This could be the case if the nanoparticles act as a Trojan horse by enhancing the transport of phenanthrene to intracellular receptors (Farkas et al., 2012). In the third scenario CeO_2 NPs could act as an antagonist and reduce the toxicity of phenanthrene. This could, for instance, be achieved by binding of phenanthrene to the NPs preventing it from entering the cells (Walker et al., 2012).

6.3 Materials and Methods

6.3.1 Test organism

Specimens of the isopod *Porcellionides pruinosus* were collected from an unpolluted field in Coimbra (Portugal). The animals were cultured in plastic boxes containing potting soil at 20 ± 2 ºC and 16/8h photoperiod, and fed with alder (*Alnus glutinosa*) leaves. The animals were kept in lab conditions for at least one month before exposure.

6.3.2 Soil and pH measurements

Standard Lufa 2.2 natural soil (LUFA-Speyer 2.2, Germany) was used as test soil. This soil is characterized as sandy loam with an organic carbon content of 2.3 ± 0.2%, pH (0.01 M $CaCl_2$) of 5.6 ± 0.4, cation exchange capacity (CEC) of 10.0 meq/100 g and water-holding capacity (WHC) of 46.5%.

Soil pH_{CaCl2} of the test soils was measured at the beginning of the toxicity tests. Spiked soils (5 ± 0.1 g) were shaken with 25 ml 0.01 M $CaCl_2$ solution for 2 hours. After settlement of the particles, the pH of the soil solution was recorded using a Consort P907 meter.

6.3.3 Test compounds and spiking of the soil

Phenanthrene supplied by Sigma-Aldrich (98% purity) was used. CeO_2 NP powder was manufactured by Antaria with a primary particle size of approximately 10-50 nm. Figure 6.1 shows the nanoparticles when deposited on a carbon coated Cu TEM grid after dispersion of 1 mg/ml in deionised water and sonication for 30 sec in a low power US bath.

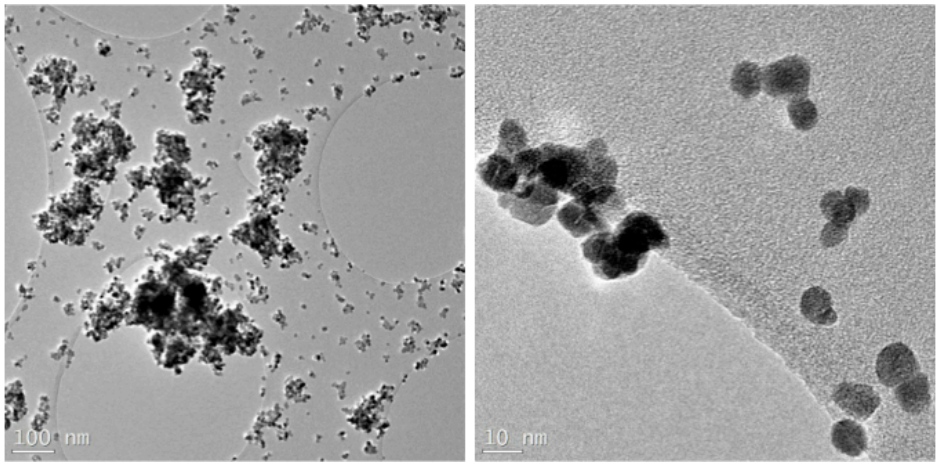

Figure 6.1: Transmission Electron Micrographs of CeO_2 NPs, when deposited on a carbon coated Cu TEM grid after dispersion of 1 mg/ml in deionised water and sonicated for 30 sec in a low power US bath. Primary particle size approx. 10-50 nm, mostly small (<100 nm) and medium sized agglomerates/aggregates (100-300 nm), some large (micron sized) agglomerates are also visible.

Soil exposures consisted of three CeO_2 NPs concentrations, namely 10, 100 and 1000 mg Ce/kg dry soil and six concentrations of phenanthrene, namely 15, 30, 60, 120, 240, and 480 mg/kg dry soil. Only three concentrations of CeO_2 NPs were tested, based on the no reproductive effect that CeO_2 NPs have shown in an earlier pilot study with earthworms at 10,000 mg Ce/kg dry soil (Lahive et al., 2014).

CeO_2 NPs were added to a large batch of dry soil to reach nominal concentrations of 10, 100 en 1000 mg Ce/kg dry soil. A total of 120 g of soil were used in each treatment for the toxicity test. Phenanthrene was dissolved in acetone (≥99% purity, Sigma-Aldrich) and added to a small amount of the CeO_2 NP spiked soil first, i.e. corresponding with 20 g dry soil for the isopod. Then, soil samples were equilibrated in closed pots for 24 hours. After equilibration, the pots were opened

to let the acetone evaporate overnight. Then, the remaining soil spiked with CeO_2 NPs, i.e. 100 g dry soil, was added to the soil spiked with phenanthrene. Soils were mixed thoroughly with a kitchen spoon and Milli-Q water was added to obtain a moisture content of 23.3% (w/w), corresponding to 50% of the WHC. Water and acetone controls were also prepared.

6.3.4 Toxicity tests

Adult isopods (15-25 mg), both male and non-gravid females, were selected for the feeding inhibition test (Silva et al., 2014; Chapter 3). The animals were exposed individually in plastic boxes containing 10 g of moist soil. For each treatment, 8 replicates were used. The boxes were incubated at 20 ± 1 °C and a light/dark regime of 12/12 h. Alder leaves previously cut in disks (Ø 10 mm) were offered as food *ad libitum*. Soil moisture content was adjusted after 7 days by weighing the test containers. Survival, food consumption (mg food/mg isopod) and changes in biomass (% of fresh weight) were evaluated after 14 days.

6.3.5 Data analysis

Concentrations causing 50% mortality (median lethal concentrations or LC50s) of *P. pruinosus* were calculated by the Trimmed Spearman-Karber method (Hamilton et al., 1977). Food consumption ratio (Cr) and biomass change (B) were calculated as:

$Cr = (W_{Li} - W_{Lf}) / W_{isop}$

$B = (W_{isopf} - W_{isop}) / W_{isop} * 100$

where, Cr is the consumption ratio (mg leaf/mg isopod), W_{Li} the initial leaf weight (mg dw), W_{Lf} the final leaf weight (mg dw), W_{isop} the initial isopod weight (mg fw), B the biomass change (%), and W_{isopf} the final isopod weight (mg fw).

Isopod consumption ratio was analysed by a two-way Analysis of Variance (ANOVA) followed by Fisher LSD post-hoc test, after log-transformation. Data homoscedasticity and normality were tested by Levene's test and the Kolmogorov–Smirnov test, respectively.

EC50 values for effect on isopod biomass change were calculated applying a 4-parameter logistic model ($Y=ymin+(ymax-ymin)/(1+(X/EC50)^{-b}$). To assess effects of CeO_2 NPs, biomass of isopods exposed only to CeO_2 NPs was analyzed by one-way ANOVA. A generalized likelihood ratio test (Sokal and Rohlf, 1995) was applied to compare EC50 values obtained for each treatment. All calculations were performed in SPSS Statistics 20.

6.4 Results

Soil pH_{CaCl2} was not affected by the addition of CeO_2 NPs or phenanthrene and ranged between 5.53 and 5.71 for *P. pruinosus.*

No mortality was observed in the control soil, while one out of eight isopods died (13%) in the acetone control. No mortality was observed for isopods exposed to CeO_2 NPs up to 1000 mg Ce/kg dry soil. Phenanthrene had a dose-related effect on isopod survival, with LC50s ranging from 110 to 143 mg/kg with no significant effect of CeO_2 NPs (Table 1).

Table 6-1: LC50 and EC50 values with 95% confidence interval for the toxicity of phenanthrene at different concentrations of CeO_2 (mg Ce/kg dry soil) to *Porcellionides pruinosus* exposed to Lufa 2.2 soil for 14 days. LC50 values were obtained with the Trimmed Spearman-Karber (TSK) method (Hamilton *et al.*, 1977) and EC50 values by logistic regression.

Compound(s)	LC50 (mg Phe/kg dry soil)	EC50 (mg Phe/kg dry soil)
Phenanthrene (Phe)	137	18.8[a]
	(101-187)	-
Phe + 10 mg Ce/kg	110	26.1 [a]
	(79-153)	-
Phe + 100 mg Ce/kg	128	17.6 [a]
	(92-176)	-
Phe + 1000 mg Ce/kg	143	31.6 [a]
	(107-190)	-

[ab] Indicate significant differences between EC50 values according to a generalized likelihood-ratio test ($X^2_{df} > 3.84$; $p < 0.05$).

-Data did not allow calculating reliable 95% confidence intervals.

Because high mortality (>50%) was observed at phenanthrene concentrations above 120 mg/kg, the surviving animals were excluded from further analysis for consumption ratio and biomass change. Consumption ratio was affected by CeO_2 NPs and by phenanthrene (Two-way ANOVA, Fisher LSD test, $p< 0.05$) (Fig. 2A). No significant interaction was found between CeO_2 NPs and phenanthrene (Two-way ANOVA, $p>0.05$).

Isopod biomass was not affect by CeO_2 NPs, and no difference between animals exposed to CeO_2 NPs and control was found (One-way ANOVA, $p>0.05$). Biomass dose-related decreased with increasing phenanthrene concentration (Fig. 2B). EC50 values for phenanthrene in the presence of CeO_2 NPs ranged from 17.6 to 31.6 mg/kg, and were not significantly different from each other according to a generalized likelihood-ratio test ($X^2_{df=1} <3.84$, $p < 0.05$) (Table 1).

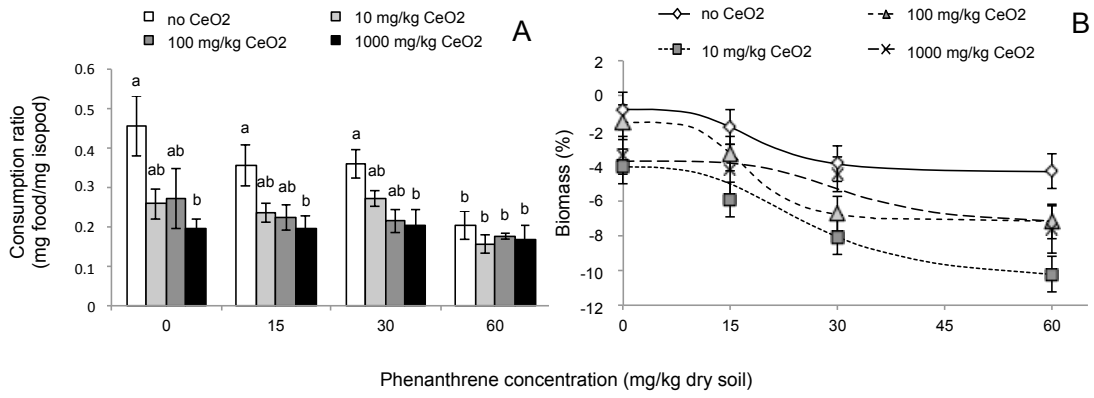

Figure 6.2: Consumption ratio (A) and biomass (B) of the isopod *Porcellionides pruinosus* exposed for 14 days to phenanthrene in the presence of CeO_2 NPs in Lufa 2.2 soil. Lines represent fit obtained with a four-parameter logistic model.

6.5 Discussion

Combined exposure to CeO_2 NPs and phenanthrene is likely to occur in the environment, because both compounds are present in diesel fuel. The use of CeO_2 NPs provides advantages in reducing emission during fuel combustion. This study showed that the release of CeO_2 NPs with phenanthrene into the

environment does not lead to an enhanced toxicity of the latter to soil arthropods. It also showed that CeO_2 NPs have low toxicity to soil arthropods.

Many components are influencing CeO_2 behaviour and its interaction with phenanthrene in soils. Soil organic matter content, for example, is an important factor affecting CeO_2 NP behaviour (Batley et al., 2013; Cornelis et al., 2011; Zhao et al., 2012). Organic matter content controls CeO_2 NPs bioavailability, as it determines the partitioning of CeO_2 NPs in the soil solution (Zhao et al., 2012). As shown by Cornelis et al. (2011), CeO_2 NPs are predominantly negatively charged in soils due to the adsorption of phosphate and organic molecules. As a result, positively charged clays attract CeO_2 NPs. The dissolution of Ce from CeO_2 NPs was very low and no levels of Ce could be detected after ultrafiltration (Cornelis et al., 2011).

The binding of phenanthrene to CeO_2 NPs in soil remains uncertain. In water, it has been shown that the binding of phenanthrene to NPs can be explained by electron donor-acceptor interactions (Fang et al., 2008; Farkas et al., 2012). Phenanthrene can bind to citrate-coated Au NPs due to the highly electronegative core material of Au NPs that attracts phenanthrene, where phenanthrene acts as an electron donor (Farkas et al., 2012). In spiked soils, however, the characterization of NPs in the presence of phenanthrene is extremely difficult, and outside the scope of this study.

So far, studies on the toxicity of CeO_2 NPs to soil invertebrates were mainly restricted to the nematode *Caenorhabditis elegans* and recently a study of CeO_2 NPs toxicity for the earthworm *Eisenia fetida* has been published (Lahive et al., 2014). Zhang et al. (2011) showed that *C. elegans* survival was affected by a concentration of 1 nM. Due to the low dissolution of CeO_2 NPs into ionic forms in nematode growth media, the authors suggested that uptake of nanosized Ce occurred. Toxicity of CeO_2 NPs with different functionalized surfaces, including positive, negative and neutral charges, has been reported in moderately hard reconstituted water (MHRW) (Collin et al., 2013). Positively charged CeO_2 NPs were found to be more toxic and showed higher bioaccumulation in *C. elegans* than negatively charged or neutral NPs. Moreover, the authors also found that the presence of humic acid decreased the toxic effects of CeO_2 NPs and this had a

greater influence on the toxicity than the NP surface charge (Collin et al., 2013). For earthworms, however, no effects on survival and reproduction were observed up to 10,000 mg/kg, although Ce body concentration increased in a dose-related manner with soil Ce concentration (Lahive et al., 2014). Because they did observe histological changes in Ce-exposed animals, the authors did not exclude a long-term effect of CeO_2 NPs.

This latter study is in agreement with our findings, where CeO_2 NPs did not show toxicity to the isopod *P. pruinosus* at high exposure concentrations. This could be explained by the much lower bioavailability of CeO_2 NPs in soils than in liquid exposure media.

This study is the first report on phenanthrene toxicity to the isopod *P. pruinosus*. A previous study evaluated the effects of phenanthrene to other isopod species using contaminated food (Van Brummelen et al., 1996). The authors found no effect on the survival of *Porcellio scaber* and *Oniscus asellus*, and a slight reduction in growth for *O. asellus* after exposure for 16 (*P. scaber*) and 47 weeks (*O. asellus*) to phenanthrene at 706 mg/kg dry food (Van Brummelen et al., 1996). For the earthworm *Eisenia veneta* 28-day LC50 and EC50 values of 134 and 94 mg/kg dry soil, respectively were reported for the effects on growth (Sverdrup et al., 2002). This LC50 was similar to the values estimated in the present study for *P. pruinosus* (110-143 mg/kg dry soil). The EC50 values regarding the isopod biomass (17.6-31.6 mg/kg dry soil) however suggest that *P. pruinosus* was more sensitive to phenanthrene than the earthworms. In addition, CeO_2 NPs did not affect phenanthrene toxicity to *P. pruinosus*.

The data supports our first hypothesis, which states that CeO_2 and phenanthrene act in an additive way. However, mixture toxicity could also be expressed only by phenanthrene toxicity as CeO_2 did not have any effect by itself nor did it affect phenanthrene toxicity up to concentrations of 1000 mg Ce/kg dry soil. This study, however, is limited by lack of information on the characterization and bioavailability of the test compounds. We suggest further studies to determine the behaviour of the mixture of CeO_2 NPs with phenanthrene and evaluate the environmental factors affecting it.

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CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

The nanotechnology has developed innumerous products that make use of metal-based nanoparticles (NPs), such as bactericidal textiles (Ag NPs), sunscreens (ZnO NPs), paints and fuel additives (CeO_2 NPs). The production of these NPs has rapidly been increasing in the last decade, but still their potential effects in the environment are not fully understood. As the soil compartment has been identified as a sink of NPs, especially the ones used in consumer products, the need for determining their toxicity to soil organisms has increased. Thus, the aim of this thesis was to assess the toxicity and bioaccumulation of these three metal-based NPs in the isopod *Porcellionides pruinosus*.

The literature review presented in Chapter 2 has clearly highlighted the importance of the characterization of both NPs and receiving environmental media. Because soil is a complex matrix, there are some limitations in properly characterizing NPs in soils (Hassellöv et al., 2008; von der Kammer et al., 2012), but still it is extremely important. For instance in Chapter 4, Transmission Electron Microscopy (TEM) images of soil extracts (i.e., soil samples dispersed in water) showed that single particles (5-8 nm) and aggregates (20-50 nm) could be found. Moreover, the behavior and speciation of metal-based NPs can be better understood if the available metal concentration in the pore water and total metal concentration in the soil are measured. Testing the ionic counterpart is also a good approach to give insight in the role of the ionic metal forms that may be released by dissolution of metal-based NPs.

In Chapter 3, the dissolution of ZnO NPs, non-nano ZnO and $ZnCl_2$ in soils was determined by measuring the total Zn concentration in porewater and by modeling the free ion concentration. ZnO NPs present relatively high solubility in soils compared to other NPs (Du et al., 2011; Wang et al., 2009). Dissolved Zn concentrations from ZnO particles increased with increasing Zn concentration in the soil. Still, the soluble salt $ZnCl_2$ presented higher levels of Zn dissolved in porewater and higher free Zn ion activities calculated by the WHAM 7 model. The partitioning of metals between soil porewater and soil particles is a result of dissolution and sorption processes. The dissolved metal ions released from NPs and the other cations present in the soil (such as H^+ or Ca^{2+}) compete for binding to the sorption sites on the soil particles (Sauvé et al., 2000; Tipping et al., 2003).

In Chapter 3, it was observed that soil pH decreased and Ca^{2+} levels in the pore water increased with increasing $ZnCl_2$ concentration in the soil. This was expected because Zn^{2+} ions compete with H^+ and Ca^{2+} for binding to the soil particles. On the other hand, for ZnO NPs (30 nm) and non-nano ZnO (200 nm), soil pH and Ca^{2+} levels in porewater depended on initial soil pH. At low and intermediate pHs (i.e., pH ~4.5 to 6), pH was increased when increasing ZnO particles concentration in soil, while at high pH (pH 7), a slight decrease in pH levels was observed when increasing ZnO concentration. It was however, also found that the behavior (i.e., dissolution, sorption) of ZnO particles was quite different from that of $ZnCl_2$.

Cations and dissolved metallic forms also compete for binding onto the membranes of biota, as described by the biotic ligand model (Ardestani et al., 2014; He et al., 2014; Thakali et al., 2006). Higher levels of dissolved cations therefore may reduce the toxicity of metals ions, leading to a protective effect from cations. In this way, soil pH can affect the toxicity of metals to soil organisms (Spurgeon et al., 2006; Waalewijn-Kool et al., 2014; Waalewijn-Kool et al., 2013). In the case of NPs, the interaction between soil pH, cation concentration and free metal ion concentration can be more complex than that, since not only dissolved ions but also nanosized particles will be present. Thus, in Chapter 3, the influence of soil pH on ZnO NPs toxicity to isopods was assessed. No evidence of a protective effect of cations could be found. Even though the behavior of ZnO particles and $ZnCl_2$ differed, the LC50 and EC50 values generally peaked at intermediate pH (Figure 7.1), independent of the Zn form tested. One possible explanation for these findings is the route of exposure in isopods. These organisms are less exposed to dissolved fractions of metals in comparison to other invertebrates, since dermal uptake (directly from soil porewater) is negligible.

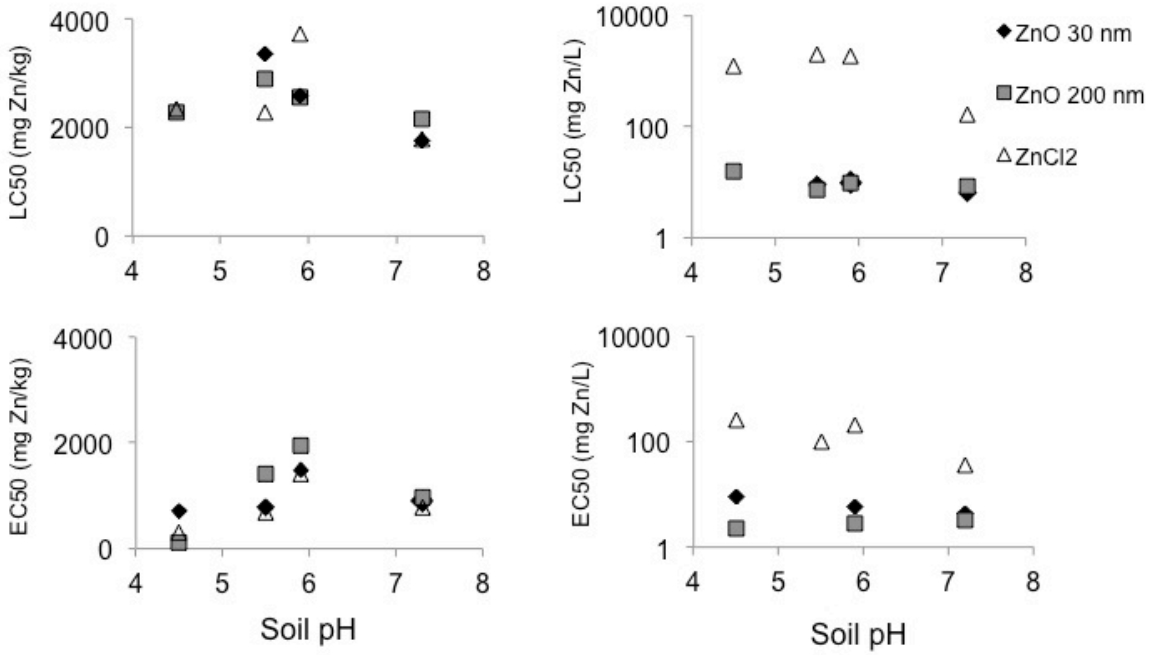

Figure 7.1: LC50 and EC50 values for the effect of ZnO NPs (30 nm), non-nano ZnO (200 nm) and $ZnCl_2$ on the survival and biomass change of the isopod *Porcellionides pruinosus* exposed to 4 soils at different pH levels. LC50 and EC50 for effects on biomass change were calculated based on total soil concentrations (mg Zn/kg; left) and porewater concentrations (mg Zn/l; right).

When LC/EC50 values were calculated based on the porewater concentration (Figure 7.1, right column), higher values were found for $ZnCl_2$. It suggested that the toxicity of ZnO NPs and non-nano ZnO could not be caused only by the dissolved Zn in porewater. The same observation was valid for Zn uptake, as Zn body concentration could not be related only to dissolved Zn concentrations. Thus, it can be concluded that Zn was also taken up as nanoparticle, probably because oral uptake is the main route of exposure for ZnO NPs in isopods.

In Chapter 4, the toxicity of Ag NPs and $AgNO_3$ was evaluated in isopods exposed via soil and food. The most sensitive endpoint was the avoidance behavior in the soil exposures. Ag NPs and $AgNO_3$ caused similar avoidance behavior with EC50s of ~14 and 16 mg Ag/kg. When exposed to Ag-spiked food, consumption ratio was significantly decreased at ~30 mg Ag/kg food. Probably, isopods are able to detected and avoid Ag in both soil or food, independent of the Ag form. This is an important finding since the avoidance behavior is a practical and fast, but yet

sensitive method for evaluating the effects of contaminants in soils (De Silva and van Gestel, 2009; Loureiro et al., 2005).

Only $AgNO_3$ significantly reduced isopod biomass upon dietary exposure. Following soil exposure, EC50s were quite similar for both Ag forms, nevertheless $AgNO_3$ caused a greater reduction in isopod biomass. These results suggest that $AgNO_3$ was slightly more toxic than Ag NPs in the feeding inhibition tests. Nevertheless toxicity could not be explained by Ag body concentrations in the isopods, since no difference was found between Ag NPs and $AgNO_3$. For the Ag NPs exposure, it is probable that Ag has been taken up as both nanosized and ionic Ag. TEM images of soil solution from spiked soils have shown the presence of both aggregates and single particles of Ag NPs. Mortality was observed in isopods exposed to $AgNO_3$, with a LC50 of ~400 mg/kg soil (nominal concentration), while Ag NPs did not affect survival up to 455 mg Ag/kg soil.

The following step was to assess the bioaccumulation of Ag NPs and $AgNO_3$ in isopods exposed via soil and food (Chapter 5). In the soil exposures, no differences in the uptake and elimination rate constants (k1 and k2), and bioaccumulation factors between Ag NPs and ionic Ag were observed. In the dietary exposures, Ag concentrations in $AgNO_3$ spiked food were higher than for Ag NPs. As a result, food consumption was lower in the $AgNO_3$ exposures, which resulted in up to 5 times lower k1 for $AgNO_3$ than for Ag NPs.

Interestingly, very low elimination rate constants were observed in all treatments. Thus, it can be concluded that low Ag elimination is independent of Ag form or exposure pathway (i.e., soil or food). Ag storage in inert granules was probably the main strategy for Ag detoxification in the isopods, as the metal granules formed in the hepatopancreas are not toxic. This is supported by the high inert fractions found in soil exposures, shown in Chapter 5. The findings from the kinetics experiments suggest that uptake, storage and elimination of Ag were similar for Ag NPs and $AgNO_3$.

When exposed to Ag NPs, it can be assumed that both nanoparticulate (NPs) and ionic Ag (Ag^+) may be taken up by the isopods via either soil or food pathways (Figure 7.2). It was assumed that oral uptake is also the main route in soil exposures, since the dermal uptake (via the pleopods) is negligible (Vijver et al.,

2006). After oral uptake, Ag assimilation is likely to take place in the gut. However, it remains unclear whether only Ag ions are assimilated or whether nanosized Ag can also be assimilated in the gut (Figure 7.2).

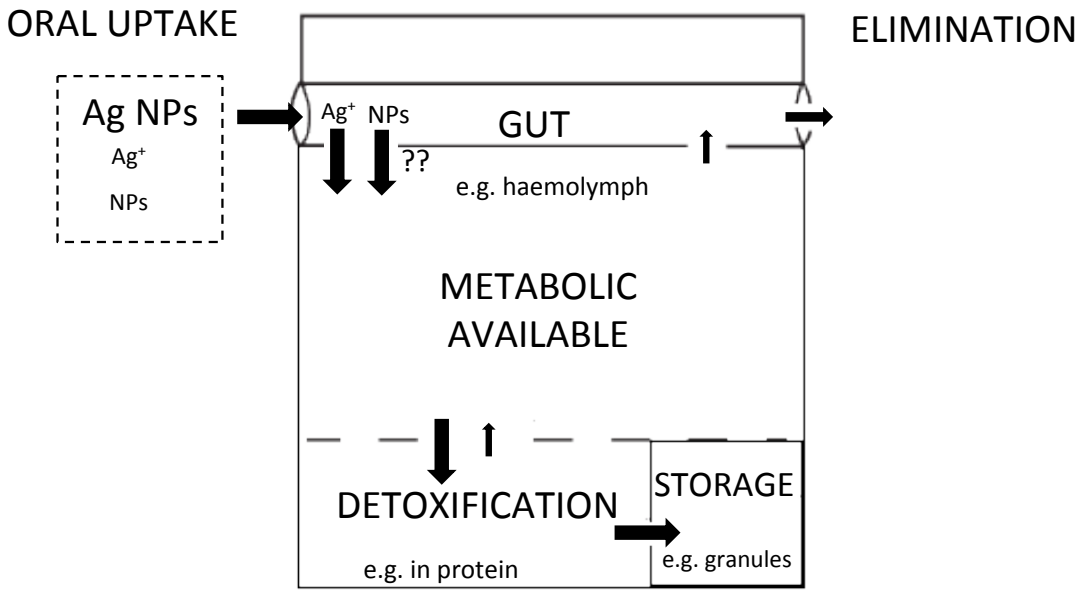

Figure 7.2: Hypothetical scheme for distribution of Ag between the active (metabolically available) and storage pools (detoxification and storage). Ag NPs are taken up orally as Ag ions (Ag^+) and Ag NPs (NPs), and assimilated in the gut. Afterwards, detoxification (e.g., binding to proteins) and storage (e.g., metal-rich granules in the hepatopancreas) are probably the main strategies for dealing with the high Ag concentration in the body. A small fraction of the Ag taken up is eliminated via the faeces. Adapted from Rainbow (2007).

When entering the body, metals are metabolically available until bound to proteins such as metallothioneins (Rainbow, 2002; Rainbow, 2007). Besides bound to metallothioneins, a second mechanism for detoxification is the intracellular deposition in metal-rich granules (Hopkin, 1990; Vijver et al., 2004).

According to Hopkin (1990), in the hepatopancreas metals can be associated to three types of granules. Different pathways are expected depending on the metal and the organism's physiology. Metals like cadmium and copper are retained in type B granules in the S-cells. The type B granules are sulphur-rich and the metals associated with these granules must have an affinity to sulphur (Hopkin, 1990). Thus, it is very likely that Ag will follow this pathway, due to its high affinity to sulphur. To support this hypothesis, a preliminary synchrotron micro-X-ray fluorescence (μXRF) analysis was performed on the hepatopancreas tissue of

isopods exposed to Ag NPs. The results indicated that Ag was present in the S-cells and related to the presence of sulphur and copper (Figure 7.3).

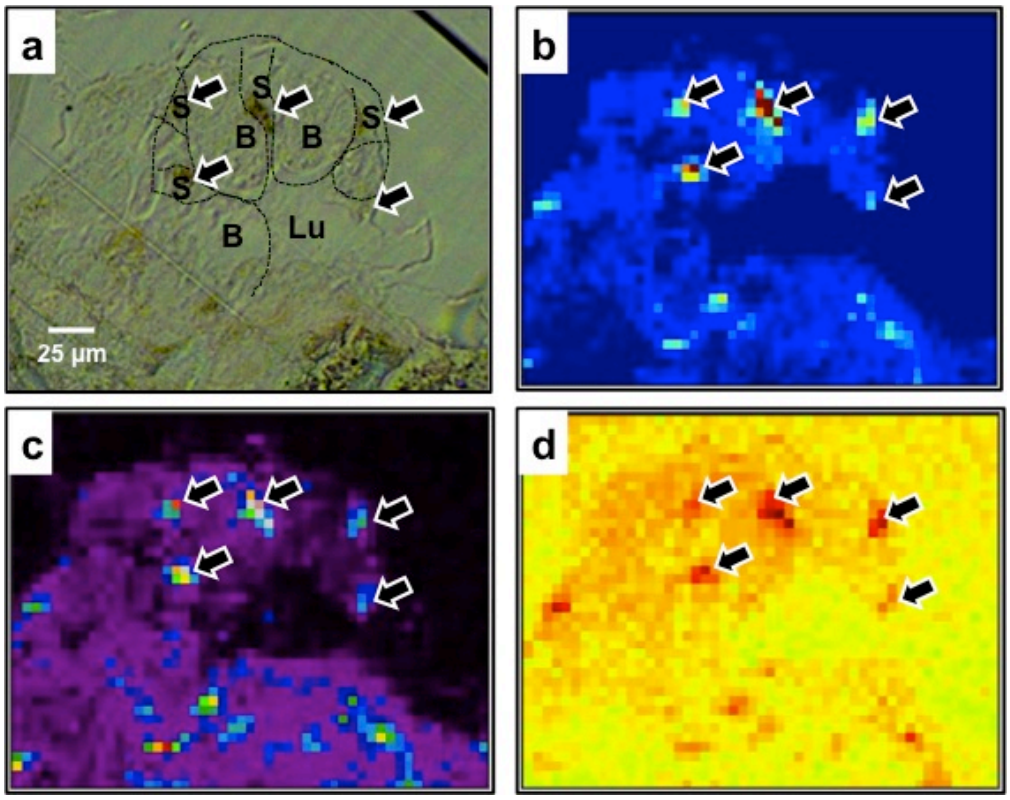

Figure 7.3: µXRF maps of element distributions in of woodlouse hepatopancreas exposed to dietary Ag-NPs. (a) Light micrograph of a transverse section. Note that the morphology of the section is unclear due to a lack of differential contrast in the unstained sections. The outlines of some of the constituent 'S'-cells (S) and 'B'-cells (B) surrounding the lumen (Lu) are approximately delineated with dotted lines. Sulphur (b), Copper (c), and Silver (d) µXRF maps acquired across the entire section depicted in the micrograph. Figure kindly provided by Claus Svendsen, Peter Kille, A. John Morgan, Kerstin Jurkschat, and Fred Mosselmans.

Since most Ag seems to be stored in the hepatopancreas, only a small fraction is probably freely available to cause toxicity (i.e., metabolically available). If similar mechanisms of storage and elimination occurred in isopods from the toxicity tests as well (Chapter 4), this can explain the lack of a relationship between toxic effects and Ag body concentrations. In accordance to Rainbow (2002), when metals are stored in detoxified forms, no relation between toxicity and body concentration can be found.

When comparing soil and food exposure, higher toxicity and bioaccumulation of Ag was observed in soil. In the feeding inhibition test via soil, EC50 for effects on biomass were 114 and 120 mg Ag/kg dry soil for Ag NPs and $AgNO_3$, respectively. In the dietary exposure to Ag NPs, no decrease in biomass was observed up to ~1500 mg Ag/kg dry food in comparison to the control. For $AgNO_3$, food exposure resulted in an EC50 for effects on biomass of 233 mg Ag/kg dry food. Higher toxicity and bioaccumulation of soil exposure has been shown in the literature for other compounds (such as metals and organic pesticides), and now it was found to be valid also for Ag NPs (Sousa et al., 2000; Vijver et al., 2006; Vink et al., 1995).

The uptake rate constants (k1) for Ag following both routes of exposure were compared using a one-compartment model, with no inert fraction, since the model with the inert fraction failed to fit the data from the dietary exposures. Even though the concentrations in food were much higher than soil concentrations, some conclusions can be drawn since body concentration (Q) is a result of the uptake constant rate and exposure concentration (i.e., 'k1' times 'exposure concentration'). For Ag NPs, k1 was about 10 times higher in soil exposures than in dietary exposures. This indicates that soil exposure will lead to higher uptake of Ag NPs than dietary exposures. As discussed in Chapters 4 and 5, the higher organic matter in food may lead to stronger binding, reducing the bioavailability in dietary exposures. Also, the decrease in consumption ratio of contaminated food by the isopods, together with the reduced bioavailability due to their aggregation on the food surface (Judy et al., 2012; Unrine et al., 2012) may explain these results.

Vijver et al. (2006) observed higher elimination rates in the isopod *Porcellio scaber* when exposed to dietary Cd and Zn in comparison to soil exposure. The authors suggested that food might sorb free or loosely bound metal fractions in the gut, which may be eliminated via the faeces. Nevertheless, no differences in the elimination rate constants of Ag NPs and ionic Ag were observed in Chapter 5. Considering the higher k1 found for soil exposures and similar k2 between soil and food, it can be assumed that soil exposure will lead to higher Ag bioaccumulation than the exposure via food.

Finally, the toxicity of CeO_2 NPs was assessed in the presence and absence of phenanthrene in soil (Chapter 6). No effects of CeO_2 NPs on isopod survival and biomass were reported up to 1000 mg Ce/kg dry soil. Moreover, no shift in phenanthrene toxicity was observed with increasing CeO_2 NP concentration in soil. CeO_2 NPs are likely to enter the soil compartment, with predicted concentrations in biosolids ranging between 0.5 and ~10 mg/kg soil (Lazareva and Keller, 2014). Although more studies are necessary to address the potential toxicity of CeO_2 NPs, their effects on soil invertebrates seem to be negligible compared to the benefits of the use as fuel catalyst in diesel (i.e., decrease of oxidation rate and emission of particulate matter).

By assessing the effects of three different nanoparticles to terrestrial isopods, it was found that toxicity varies depending on the metal present (Table 7.1). These results can be used as a screen to identify which NPs are more toxic to other soil invertebrates presenting similar exposure routes as isopods. Among the tested compounds, Ag NPs and the ionic counterpart $AgNO_3$ were the most toxic to isopods. Even though the concentrations used in the tests were several orders of magnitude higher than the predict concentrations for Ag in the environment, isopods accumulated high levels of Ag and hardly eliminated it. Therefore, it may be a matter of concern for these organisms when exposed to lower Ag concentrations but during extended periods of time. The high accumulation potential of Ag in isopods may also lead to a potential risk of food chain transfer of Ag to isopods' predators.

Table 7-1: LC50 and EC50 values for the effects on the survival and biomass of the isopod *Porcellionides pruinosus* for Zn (as ZnO NPs, non-nano ZnO, and $ZnCl_2$), Ag (Ag NPs and $AgNO_3$) and CeO_2 NPs. The isopods were exposed to Lufa 2.2 soil for 14 days in feeding inhibition tests. LC50s were calculated using Probit analysis, while EC50s were calculated with a 4-parameter logistic curve. 95% confidence intervals are presented in brackets.

	Unit	LC50	EC50 biomass
ZnO NPs	mg Zn/kg soil	3361 (2593-4839)	788 (117-1458)
Non-nano ZnO		2984 -	1405 (670-2141)
$ZnCl_2$		2292 (1698-3229)	687 (332-1042)
Ag NPs	mg Ag/kg soil	>455	120 -
$AgNO_3$		394* (-)	114 -
CeO_2 NPs	mg Ce/kg soil	>1000*	>1000*

*Based on nominal concentrations.

The dissolution of NPs releases free ions that partition between soil particles and porewater. Usually, the ionic fraction is suggested to be the main responsible for the toxicity of metals or metal-based NPs to soil organisms, although there is no consensus among studies. The findings of this thesis do not completely support this hypothesis. It is difficult to define NP speciation in soils and thus to determine the fractions causing toxicity. Independent on whether NPs caused toxicity or not, the results suggest that ZnO and Ag have been taken up as nanosized particles by the isopods. It was supported by the low ZnO dissolution in comparison to ionic Zn (Chapter 3) and the presence of single particles and aggregates of Ag NPs shown by TEM images (Chapter 4).

Over the last decades, isopods have been considered to be a good indicator for metal pollution in soils. The model species used, the isopod *Porcellionides pruinosus*, was found to be suitable for evaluating the toxicity and bioaccumulation of metal-based NPs. Therefore, this species showed a high potential to be used as

a test species for assessing the contamination and toxicity of NPs in soil environments.

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